# THE CLINICAL APPLICATION OF HORMONE ASSAY

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BY

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WITH A FOREWORD BY

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THIS BOOK
IS DEDICATED TO
PROFESSOR
GUY FREDERIC MARRIAN
TO WHOM THE SCIENCE
OF ENDOCRINOLOGY
OWES SO MUCH

# FOREWORD

## by J H GADDUM ScD FRS

"When you can measure what you are speaking about and express it in numbers, you know something about it, but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind."—LORD KELVIN.

MANY of the most active physicians are rather specially interested in the glands of internal secretion. It is fascinating to think that these small specialised organs should produce such widespread effects and that some of their secretions should be associated with the strongest human emotions. Various dramatic diseases are directly due to derangements of these glands, and most diseases affect them to some extent. Methods of assessing their activities have therefore attracted widespread interest. Direct observations of their effects on the patients themselves can sometimes provide all the information that is needed but such observations are often not enough, and an army of enthusiastic research workers has therefore sought to devise methods of estimating hormones and their metabolites in the blood and urine.

Various methods of diagnosing pregnancy by biological tests for gonadotrophins in urine were shown to give reliable results without elaborate precautions. It was natural to suppose that similar simple tests for other hormones would also give information of clinical importance but the results have on the whole been confusing. Some of the reasons for this have now become apparent. The pregnancy tests are successful because pregnancy causes enormous changes in the hormones in the urine, and quantitative estimates of the amounts present are unnecessary for the diagnosis. For other purposes quantitative results are needed and it is only lately that adequate quantitative methods have been devised.

It was thought at one time that it would be possible to estimate the total activity of various kinds in blood or urine. For example urine was known to contain several substances which produced oestrogenic effects in small animals. Extracts of urine were therefore injected into mice and the smallest quantity to produce oestrus was called a mouse unit, so that the oestrogenic activity of urine could be estimated in mouse units per litre. There are several reasons why this convenient device gave results which were not reproducible. Since mice were found to vary the dose for the average mouse was measured. Since one colony of mice gave different results from another and the same colony gave different results on different occasions, standard preparations of oestrogens were used in each

experiment, but even this precaution was not enough. Human urine contains a number of different substances with œstrogenic effects and the ratios of their activities vary enormously according to the exact design of the experiment. By one technique, for example, œstrone is one hundred times as active as œstriol and by another technique the activities of these two substances are about equal. If one of these substances were used as a standard to test urines whose activity was mostly due to the other substance the results in one laboratory might be one hundred times as large as the results in another. Such findings convinced those concerned that the measurement of the total activity of an extract had no real meaning and that progress could only come from the estimation of the concentration of each of the substances contributing to the effect. This can be achieved by first separating the extract into fractions, each containing one active substance, and then estimating the concentration in each fraction by bio assay, using a standard preparation of the appropriate substance, but in the case of the œstrogens in urine quicker and more precise results can be obtained by applying colorimetric methods to the fractions. The important point is that it is necessary to estimate the concentration of single substances and not the total activity; this is true not only of the œstrogens but of all assays.

Chemical methods of assay are replacing biological methods. They are generally quicker and more precise and, if they can be shown to give an estimate of the concentration of a single substance, this estimate is just as valuable as a biological estimate. Some biological methods are still more sensitive than the best chemical methods, but the latter have improved much recently. Some of the simpler hormones can now be estimated in any department of clinical chemistry which has enough trained staff, but each assay involves much work. There is no immediate likelihood that it will be possible to estimate all the polypeptide hormones by simple chemical procedures, but some of them can be estimated by their effects on isolated tissues or tissue slices, and it is probable that the use of whole animals for hormone assays will eventually be abandoned.

Some hormone assays are an aid to diagnosis and a guide to treatment and can be regarded as routine procedures which should be generally available. For example, estimates of human chorionic gonadotrophin may assist in the diagnosis of pregnancy, chorion epithelioma or hydatidiform mole, and estimates of sympathomimetic amines may assist in the diagnosis of adrenal medullary tumours. The meaning of estimates of other hormones however is often not immediately obvious, and the purpose of most assays is to advance knowledge rather than to guide the doctor in the treatment of individual cases.

It is still important that researches in this field should be carefully planned with full knowledge of the limitations of the methods used, most of which are laborious and costly, and that the programme

should not be seriously interrupted by demands for isolated observations on interesting cases not connected with the main investigation It has sometimes happened in the past that clinicians have employed biochemists to estimate hormones by some published method, and that the biochemists have been persuaded to earn their living by using methods in which they have no faith, in researches which seem to them to be badly planned Such arrangements have been unfruitful

Attempts have been made to establish central laboratories to carry out hormone assays for the doctors in a district Such laboratories have at present only a limited use in connection with such things as pregnancy diagnosis Their scope may widen with the advance of knowledge but there is a danger that much of the money and effort spent on such laboratories may be wasted on unnecessary assays

It is probable that all necessary hormone assays will eventually become part of the routine work of departments of clinical chemistry, but in the immediate future real progress is most likely to come from physicians working in the laboratories on problems arising in the wards Time has justified what Sir Thomas Lewis said about the importance for medicine of the work of such people to whom he gave the name of clinical scientists This book provides many examples of the kind of work they can do

A few years ago most of the best work in clinical endocrinology was being done in the U S A More recently however important work on quantitative methods has been done in Great Britain Dr Loraine is a member of the staff of the Clinical Endocrinology Research Unit (C E R U ) which the Medical Research Council established in Edinburgh in 1946 and it is natural that his book should lay special emphasis on the work of this unit Most such units have a single full time director but C E R U is directed by a committee consisting of the professors of biochemistry gynæcology pharmacology surgery and therapeutics This has meant that several departments have collaborated in support of a unit which belongs to them all The Medical Research Council has allowed the unit to work for long periods on the development of reliable methods without demanding quick publication This policy has produced several new methods which are likely to add much to our knowledge of disease in the years to come This book gives a clear and authoritative account of this work and of the great mass of other work in this field in all parts of the world No clinical endocrinologist can afford to be without it all who are interested in the clinical application of hormone assays will find it valuable It gives a general account of the methods available, without wearisome details and a full discussion of the ways in which these assays may be of value to those responsible for the care of patients

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The Clinical Endocrinology Research Unit has been very fortunate throughout the years in being able to collaborate closely with investigators in various hormone laboratories in Europe especially with those in Stockholm and Geneva It is certain that with the passage of time this type of international collaboration will increase and should contribute greatly to further progress in clinical and experimental endocrinology Two examples may serve to illustrate the value of the international approach to the subject The first of these is the series of Colloquia on Endocrinology' held in London under the auspices of the Ciba Foundation At these Colloquia it has been possible for research workers from many nations to meet and to discuss common problems in a pleasant and informal atmosphere The second example is provided by the Gonadotrophin Club which was formed in 1953 held its first meeting in Geneva in that year and has subsequently met in Birmingham in 1955 and in London in 1957 These meetings have been attended by colleagues from various laboratories in different parts of the world and have enabled those particularly interested in the assay of gonadotrophins to exchange ideas and to plan collaborative research

I should like to take this opportunity of expressing my thanks to the many people who have helped me in the preparation of this book I am especially grateful to Professors J H Gaddum John Bruce D M Dunlop R J Kellar and G F Marrian—Directors of the Clinical Endocrinology Research Unit Edinburgh—for their interest encouragement and co operation the book was written at the suggestion of Professor R J Kellar who felt that such a work might be of value to both clinicians and laboratory workers

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# PREFACE

This book has been written with a threefold purpose firstly, to review the advantages and limitations of existing hormone assay methods and where possible to indicate the most suitable method or group of methods for use in clinical studies secondly to discuss the application of hormone assay procedures to clinical problems and to examine the value of these estimations in the diagnosis prognosis and treatment of disease in man and finally to suggest the fields of investigation to which assay methods might in the future be profitably applied

The subject matter deals mainly with pituitary placental adrenal and gonadal hormones a short chapter on insulin is also included Consideration has not been given to the parathyroid hormone or to the gastro intestinal hormones because the methods for the quantitative determination of these substances in body fluids are not yet in a sufficiently advanced state to merit their use in clinical investigations A section on the thyroid has not been included because it was felt that although the estimation of the concentration of protein bound iodine in blood gives a reasonably reliable indication of the activity of this gland in health and disease assay methods for the thyroid hormones themselves are not at present suitable for clinical application

The field of hormone assay has been expanding so rapidly in recent years that it is clearly impossible to quote all the relevant literature An attempt has therefore been made to present an out line only of the work done prior to 1947 and to concentrate more particularly on the literature published between 1947 and 1957

In writing this book it has been a great privilege for me to express the views which the Edinburgh group as a whole share with regard to the clinical application of hormone assays One of these views which deserves special mention concerns the question of methodology We have always believed that in clinical studies only the best of the available techniques should be used and that if unreliable assay methods are employed the results obtained are liable to be mis leading Fortunately within recent years assay methods for many of the hormones have been greatly improved and it is now possible in many cases to employ techniques which satisfy the reliability criteria discussed in Chapter I

The Clinical Endocrinology Research Unit has been very fortunate throughout the years in being able to collaborate closely with investigators in various hormone laboratories in Europe especially with those in Stockholm and Geneva It is certain that with the passage of time this type of international collaboration will increase and should contribute greatly to further progress in clinical and experimental endocrinology Two examples may serve to illustrate the value of the international approach to the subject The first of these is the series of Colloquia on Endocrinology held in London under the auspices of the Ciba Foundation At these Colloquia it has been possible for research workers from many nations to meet and to discuss common problems in a pleasant and informal atmosphere The second example is provided by the Gonadotrophin Club which was formed in 1953 held its first meeting in Geneva in that year and has subsequently met in Birmingham in 1955 and in London in 1957 These meetings have been attended by colleagues from various laboratories in different parts of the world and have enabled those particularly interested in the assay of gonadotrophins to exchange ideas and to plan collaborative research

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reported in Chapters II and III could not have been obtained without expert technical assistance in the laboratory, and it gives me great pleasure to acknowledge the help of my technicians, particularly of Miss M. A. Mackay.

Finally, I should like to express my gratitude to my publishers, Messrs E. & S. Livingstone, for their very helpful collaboration and advice throughout the preparation of this book.

JOHN A. LORAINE

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JOURNALS

*Acta Endocrinologica* Copenhagen Figs 6 7 8 9 11 12 13 56 57 62 63

*Journal of Biological Chemistry* Fig 50

*Journal of Clinical Endocrinology and Metabolism* Figs 1 2 3 4 5 30 31 51 5 58

*Journal of Endocrinology* Figs 17 53 64 Tables XIII XVI

*Journal of Obstetrics and Gynaecology of the British Empire* Figs 18 19 o 1 22 26 27 28 9 47 48 49 Tables V VI

*Journal of Physiology* Table XIII

*Klinische Wochenschrift* Table XVII

*Lancet* Figs 35 36 37 38 39 40 41 42 43 53 Table XV

*Vitamins and Hormones* Tables I II III IV XIX

BOOKS

Ciba Foundation *Colloquia on Endocrinology* London Churchill Figs 16 23 24 25 Table X

Dorfman R I and Shipley R A *The Androgens* New York Wiley London Chapman & Hall Table XVIII

Emmens C W ed *Hormone Assay* New York Academic Press Table XI

Euler U S v *Noradrenaline* American Lecture Series No 261 Springfield Ill Thomas Table XX

Gaddum J H ed *Polypeptides which Stimulate Plain Muscle* Edinburgh Livingstone Table XII

Smith R W Gaebler O H and Long C N H ed *Hypophyseal Growth Hormone Nature and Actions* New York Blakiston Division McGraw Hill Book Co Fig 66

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## CHAPTER I

# *General Principles in Hormone Assay*

ASSAY methods for hormones and their metabolites can be divided into two groups—*biological methods* and *chemical methods* Bio assays are often more sensitive and more specific than chemical assays but the latter are usually more precise, less laborious, less expensive, and much more suitable for routine use in the clinical field It can be confidently predicted that in the future chemical assay procedures will largely replace bio assays for the quantitative determination of hormones and their metabolites in body fluids

At the time of writing biological assays must still be used for the estimation of pituitary gonadotrophins, human chorionic gonadotrophin (HCG) thyrotrophin, adrenocorticotrophic hormone (ACTH), growth hormone, prolactin posterior pituitary hormones, thyroid hormones, parathyroid hormone and insulin The chemical determination of these hormones in blood and urine is not possible at the present time Chemical assay methods should now be used for the determination of œstrogens progesterone and its metabolites corticosteroids and 17 ketosteroids In the case of adrenaline and noradrenaline, chemical methods of estimation have recently become much more reliable and should probably now replace bio assays in clinical studies

As indicated in the title this chapter deals mainly with matters of general interest to those concerned with the estimation of hormones for clinical purposes In order to explain some of the terms which will be used in Chapters II to VIII a section dealing specifically with bio assay is also included

## ESSENTIAL REQUIREMENTS IN HORMONE ASSAY PROCEDURES

This subject has been reviewed by various workers, including Borth (1952) Marrian (1955) Loraine (1957 *a*)

# CONTENTS

means perfect in design, may give a reasonably good indication of the losses which occur during the *performance of the* method

It is a matter of great difficulty to make a definite statement with respect to the permissible limits of accuracy in a given steroid method This subject has been recently discussed by Marrian (1955), who suggested that a method might be regarded as satisfactory from the quantitative point of view when the recovery of added pure steroid was 75 per cent or more Marrian (1955) has also emphasised the necessity for investigators *to determine in their own laboratory* the accuracy of a given method this should always be done before the technique is used for investigations in the clinical field

In hormone assays depending on biological methods, recovery experiments of the type described above are often very laborious and this fact has prevented many workers from employing them as a means of assessing the accuracy of a method Recently however particularly in the case of ACTH and of the pituitary gonadotrophins data derived from such experiments have been reported in the literature (Sydnor and Sayers 1952, Loraine, 1957 *b*) It is to be hoped that in the future similar information will be published for the other pituitary hormones

(*b*) PRECISION [1]—An estimate of the *precision of a chemical* assay method can be obtained by carrying out multiple determinations on the same sample of blood or urine or by conducting multiple recovery experiments with the same concentration of the added compound Precision is usually expressed as the standard deviation of replicate determinations

As in the case of accuracy it is very difficult to offer any definite opinion as to what degree of precision is acceptable in a given assay method Marrian (1955) has suggested that for steroid methods, a standard deviation of $\pm 10$ per cent at optimal steroid concentrations is the best that can reasonably be expected while at sub optimal concentrations, it may be necessary to be satisfied with a standard deviation of $\pm 25$ to 30 per cent

[1] It should be emphasised that the terms accuracy and precision when applied to assay methods should not be regarded as synonymous The term precision refers essentially to the reproducibility of a method and should only be used when repeated estimations have been made on the same sample of blood or urine

and Brown *et al* (1957) It is generally agreed that the efficiency of a given method depends on two main factors—its *reliability* and its *practicability*

## 1 'Reliability Criteria' for Hormone Assays

According to Borth (1952) there are four such criteria These are *accuracy, precision, specificity* and *sensitivity* Borth (1952) has stated that, in assessing the overall value of a given assay procedure, the four criteria are of equal importance This conclusion appears to be justified in view of the fact that the criteria may vary greatly in the case of individual methods Thus a method which shows a high degree of precision may not be highly specific, while a specific, accurate and precise technique may not be sufficiently sensitive for use in a particular set of circumstances

Throughout this book the term *reliability*, when applied to a method, will be used in a general sense to embrace the four concepts of accuracy, precision, specificity and sensitivity

(*a*) Accuracy —This term may be defined as the nearness with which a given analytical result approaches the 'true' result The accuracy of a quantitative method can be studied by means of 'recovery experiments' in which determinations are made on the material being analysed before and after the addition of known amounts of the substance under investigation Results are usually expressed in terms of the percentage of the added compound recovered Accuracy can also be estimated by procedures involving radio active compounds, it is probable that in the future techniques of this kind will become increasingly popular

Some of the problems involved in evaluating the accuracy of the various steroid methods have been discussed by Marrian, (1955) Ideally recovery experiments should be conducted by adding to blood or to urine pure steroids in the forms in which they are normally present Unfortunately this is seldom practicable because the majority of the steroids occur in body fluids in the conjugated form and in most instances these conjugates are very difficult to obtain for investigative purposes Accordingly, most workers have adopted the expedient of carrying out recovery experiments in which known amounts of the pure unconjugated steroids are added to blood or urine Experiments of this type, although by no

non specific reactions are used must largely depend on the techniques employed for the purification and fractionation of the crude extracts

(*d*) SENSITIVITY —This term may be conveniently defined as the minimum amount of a substance which can be detected by a particular method As will be seen in later chapters one of the chief difficulties in the estimation of many hormones in body fluids arises from the fact that available assay methods are not sufficiently sensitive to detect the small amounts of material present This difficulty is particularly marked in the case of the pituitary hormones but is also found with certain of the steroid hormones such as progesterone

Various attempts have been made to increase the sensitivity of assay methods for pituitary hormones in blood and urine One of these which has been used by Coppedge and Segaloff (1951) for the assay of urinary prolactin and by Gray and Parrott (1953) for the estimation of plasma ACTH, consists of the administration to the test animals of a so-called booster dose of the hormone in order to bring the readings on to the working range of the dose effect curve Procedures of this kind have not gained general acceptance and it is unlikely that they will continue to be used in clinical studies It is probable that in the future assay methods for pituitary hormones will be made more reliable by developing more satisfactory techniques for the extraction of these hormones from blood and urine and by administering such concentrates to the experimental animals

## 2 Practicability of Hormone Assays

Under this heading are included such factors as *speed cost* and the *skill* required in the performance of a method It is obvious that a method which satisfies the most stringent criteria of reliability may be quite unsuitable for routine use in the clinical field This would occur if as is the case with many biological assays the technique were so laborious that many days were required before a result was obtained Such a situation would also arise if the cost per estimation in terms of chemicals technical assistance animals and equipment were very high or if the method itself were so complicated that special skill on the part of the operator was required in order to obtain reproducible results For further information on the question

In biological assays the labour involved in conducting replicate determinations is very considerable, and for this reason few investigators have so far attempted to estimate precision in this way Methods which have been employed for calculating the precision of such assays are described below

(c) SPECIFICITY —In chemical assay methods specificity can be defined as the determination of one chemical entity to the exclusion of others, in bio assays the term refers to the deter mination of one physiological activity to the exclusion of others Usually the specificity of a method depends on the cumulative evidence that the technique measures what it is supposed to measure and nothing else

Some of the factors which must be considered in assessing the specificity of a chemical assay method have recently been discussed by Brown *et al* (1957) These workers attempted to evaluate the specificity of the method developed by Brown (1955) for the estimation of œstriol, œstrone and œstradiol 17$\beta$ in human urine (see p 164) They concluded that, for the majority of urines from normal women during the menstrual cycle, normal men and normal post menopausal women, there was good evidence to support the view that the substances being determined were indeed œstriol, œstrone and œstradiol 17$\beta$ This conclusion was based on the following four kinds of evidence (i) The known high specificity of the Kober re action on which the final determination in the Brown method depends, (ii) the chromatographic behaviour of the urinary œstrogen fractions on alumina adsorption columns, (iii) the behaviour of these fractions in studies involving countercurrent distribution, and (iv) the comparison of the results obtained by chemical assay on the one hand and by bio assay on the other

Marrian (1955), Borth (1956) and others have emphasised that most of the methods used for the final determination of steroids in blood or urine extracts are not of themselves specific For example, the Zimmermann reaction is not specific for 17 ketosteroids, the production of a yellow colour with sulphuric acid is not specific for 5$\beta$ pregnane 3$\alpha$ 20$\alpha$ diol (pregnanediol), and the Porter Silber reaction (see p 259) is not specific for the 17, 21 dihydroxy 20 keto corticosteroids Accordingly, the specificities of methods in which such

with reasonable success in laboratories at the Mayo Clinic and Geneva (Albert and Berkson, 1951, Borth *et al*, 1957), but in Edinburgh could not be made the basis of a reliable quantitative assay

(*b*) The assay method for ACTH based on the depletion of adrenal ascorbic acid in hypophysectomised rats had a relatively high degree of precision when used by Sayers *et al* (1948) in Salt Lake City and by Taylor *et al* (1953) in Edinburgh but was much less satisfactory in the hands of investigators in certain other centres in the United Kingdom and the United States

The dependence of hormone assays on the strain of animal employed is important from the practical point of view It makes it advisable that, prior to the establishment of a given method for clinical purposes an investigator should satisfy himself that the strain which he proposes to use is indeed suitable for his purposes Time spent in assessing the suitability of animal strains will not be wasted and may save much unnecessary expense

It is the author s opinion that if at all practicable, hormone assays should be conducted with animals bred in the investigator s own laboratory under environmental conditions which are as constant as possible Animals purchased from dealers tend to be much less uniform, and for this reason frequently give unsatisfactory results It is almost certain that many of the difficulties reported by investigators in obtaining valid bio assays result from the fact that animals bought from dealers have been used A good example of this point is provided by the differing experiences of Albert (1956) at the Mayo Clinic and of Loraine and Brown (1954 1956) in Edinburgh with the bio assay method for pituitary gonadotrophins in urine depending on the enlargement of the uterus in intact immature mice (see p 27) Albert (1956) who bought his animals from dealers could not obtain reproducible results with this test, whereas in the hands of Loraine and Brown (1954, 1956), who used animals bred in the laboratory, the method has yielded satisfactory results over a period of approximately seven years In view of the importance of a uniform animal strain in the performance of the mouse uterus test, little quantitative significance can be attached to the results of assays in which animals obtained from diverse sources are used

of the practicability of hormone assays the reader is referred to articles by Borth (1952), Borth and de Watteville (1952), Marrian (1955) and Albert (1956)

It must be emphasised that, although the factors listed above are of importance in the clinical application of hormone assays the practicability of a given method should always be dictated by its value in the diagnosis of disease Thus even a very time consuming procedure such as that recently described by Ayres *et al* (1957) for the estimation of urinary aldosterone (*see p 261*) may have a relatively wide clinical application provided that the information obtained by the use of such a method justifies the labour involved in its performance

## HORMONE ASSAYS BY BIOLOGICAL METHODS

A general discussion on the mathematical methods employed in biological assay is outside the scope of this book For information on this subject, on which a large literature has accumulated in recent years the reader is referred to publications by Thayer (1946), Emmens (1950) Burn *et al* (1950) Finney (1952) and Gaddum (1953 *a*, *b*) The present section of this chapter is concerned with certain aspects of bio assay which in the opinion of the author, are especially relevant to the estimation of hormones in body fluids The following factors will be considered

1 Strain of animal
2 Standard preparations
3 Types of bio assay
4 Design of bio assays
5 Calculation of errors

### 1 Strain of Animal

This factor is of considerable importance in hormone assays There are numerous instances in the literature of methods which give satisfactory results in some animal strains but not in others Two examples may serve to illustrate this point

(*a*) The assay method for HCG depending on the production of ovarian hyperæmia in intact immature rats has been used

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A general discussion on the mathematical methods employed in biological assay is outside the scope of this book For information on this subject, on which a large literature has accumulated in recent years the reader is referred to publications by Thayer (1946) Emmens (1950) Burn *et al* (1950), Finney (1952) and Gaddum (1953 *a b*) The present section of this chapter is concerned with certain aspects of bio assay which in the opinion of the author, are especially relevant to the estimation of hormones in body fluids The following factors will be considered

1 Strain of animal
2 Standard preparations
3 Types of bio assay
4 Design of bio assays
5 Calculation of errors

### 1 Strain of Animal

This factor is of considerable importance in hormone assays There are numerous instances in the literature of methods which give satisfactory results in some animal strains but not in others Two examples may serve to illustrate this point

(*a*) The assay method for HCG depending on the production of ovarian hyperæmia in intact immature rats has been used

post menopausal women (see Chapter II) This extract has been termed human menopausal gonadotrophin' (HMG) and it is possible that in the future HMG or some preparation like it will become an international standard for the comparative assay of the urinary gonadotrophins excreted by non pregnant human subjects

The standard preparations listed above differ considerably in their present sphere of usefulness Those for the various steroid hormones are seldom used in clinical studies as these substances should now be determined by chemical rather than by biological methods of assay On the other hand standards for the pituitary and placental hormones will certainly continue to be used for many years in view of the great difficulties involved in the development of chemical assay methods for these substances

Standard preparations for the hormones have been made from various sources Thus those for thyrotrophin, ACTH prolactin and the posterior pituitary hormones are derived from animal pituitary tissue while that for HCG is derived from pregnancy urine As emphasised by Loraine (1957 *a*) this fact may cause difficulties when extracts prepared from human blood are assayed in terms of standard material prepared from sources other than blood For example Diczfalusy and Loraine (1955) found that under certain circumstances serum samples of HCG could not be assayed against the standard while a similar conclusion was reached by Parrott (1955) in the case of ACTH Difficulties of a similar nature will almost certainly arise when blood extracts containing thyrotrophic and growth promoting activities are assayed against the corresponding standards prepared from anterior pituitary tissue *Accordingly, it is essential for investigators to determine* by statistical methods whether a given assay procedure which they propose to use for estimations in blood satisfies the recognised criteria of validity as described by Gaddum (1953 *a*) Only assays which satisfy such criteria should be employed in clinical studies

### 3 Types of Bio assay

Hormone assays are of two main types

(*a*) Assays depending on graded effects
(*b*) Assays depending on quantal effects

## 2 Standard Preparations

In 1927 Trevan noted that the minimum dose of a substance producing an effect in one animal colony was liable to differ markedly from that in another, and that even in the same colony maintained under environmental conditions which were controlled as rigidly as possible, wide variations in sensitivity could occur at different times of the year Trevan's observations have since been amply confirmed, and it is now generally agreed that the expression of results in 'animal units' without reference to a standard is a very unreliable procedure from the quantitative point of view Gaddum (1953 *a*) has stated that the error of such estimations may be threefold or more

In the past it has been necessary to express results of many hormone assays in terms of 'animal units,' and workers have generally defined the unit as the amount of material required to produce a given effect Examples of arbitrary units which should no longer be employed are the 'mouse uterine unit' for follicle stimulating hormone (FSH),[1] the 'rat ovarian hyperæmia unit for HCG and the Junkmann Schoeller unit for thyrotrophin Within the last two decades standards have been established for the majority of the hormones, this has enabled investigators to report results in international units and has also made it possible to compare directly figures obtained from different laboratories The use of 'animal units' in assays of hormones for which international standards are available is to be deplored, it is regrettable that publications still appear in which the data are presented in this way

At the time of writing international standards are available for thyrotrophin, ACTH, prolactin, posterior pituitary hormones HCG, pregnant mare s serum gonadotrophin (PMSG) insulin, œstrogenic hormones, progesterone and androgens Standards have not yet been established for growth hormone or for parathyroid hormone A standard preparation is urgently required for the assay of pituitary gonadotrophins in human urine Recent work by Loraine and Brown (1956) has shown that urinary gonadotrophins from normal men and from normally menstruating women can be assayed in terms of an extract prepared from the urine of menopausal and

[1] As will be pointed out in Chapter II the mouse uterus test is not specific for FSH but measures a mixture of follicle stimulating and interstitial cell stimulating activities

or a four point design[1] Assays depending on a six point design[1] are more reliable than either of the preceding but are too cumbersome for use in the clinical field

(*a*) (TWO + ONE) DOSE ASSAYS (THREE POINT ASSAYS) —This is the simplest design which is acceptable for routine use Three groups of animals are used two groups receive the standard and one the unknown preparation The dose of the unknown should have an effect intermediate between the two doses of the standard The design can be used in assays depending either on graded or on quantal responses The three point assay provides no information regarding either slope difference (parallelism) or curvature such tests of validity can be performed only when more complex designs are employed

Because of their simplicity three point assays have been much used in routine clinical studies In the opinion of the author this is justifiable if at an early stage of the investigation, more complex designs have been employed in order to establish that the assay in question satisfies the recognised criteria of validity

(*b*) (TWO + TWO) DOSE ASSAYS (FOUR POINT ASSAYS) —This design was first described by Gaddum in 1933 and since then it has been much used in quantitative work Four groups of animals are employed two groups receive the standard and two the unknown preparation The design can be applied to assays depending either on a graded or on a quantal response Four point assays enable the investigator to calculate whether or not the log dose response curves of S and U differ significantly in slope if lack of parallelism is demonstrated the assay is invalid and the result has no meaning The four point design can be conveniently used in the majority of hormone assays and is probably the formula of choice for clinical studies

## 5 Calculation of Errors

There are many ways of estimating the precision of bio assays These have been reviewed by Finney (1952) Gaddum (1953 *a* *b*) Diczfalusy (1954) and others One of the most convenient methods is to express the errors of the various tests in terms of the index of precision (λ) and this procedure will be adopted in subsequent chapters

[1] Three point design – two doses of S and one of U
Four point design = two doses of both S and U
Six point design = three doses of both S and U

(*a*) GRADED EFFECTS (MEASURED EFFECTS) —In this type of assay the effect of the hormone on each animal is measured The majority of hormone assays fall into this category Familiar examples are the estimation of HCG by its ability to cause enlargement of the prostate in intact immature rats (p 71), the assay of growth hormone by its effect on body weight in hypophysectomised rats (p 127) and the determination of prolactin by its action in increasing the weight of the crop gland in pigeons (p 135)

(*b*) QUANTAL EFFECTS —In hormone assays of this type the result depends on the percentage of animals which show some definite response such as œstrus or ovulation The effect produced in each animal is said to be quantal or all or none' (Gaddum, 1933) Examples of hormone assays depending on quantal responses are the test for œstrogenic activity based on vaginal cornification in oophorectomised rodents (p 160), the ovarian hyperæmia test for HCG (p 67) and the assay method for gonadotrophins depending on the expulsion of spermatozoa in amphibia (p 68)

Quantal assays are less suitable as routine procedures than are assays depending on graded effects The reason for this is twofold In the first place the statistical methods involved in calculating the error of such tests are often complicated and laborious Secondly it has been shown by Gaddum (1933 1953 *a*) and by Perry (1950) that, other things being equal the number of animals required in a quantal assay for any given degree of precision is approximately double that required in assays based on a graded response

## 4 Design of Bio assays

It is generally agreed that the most satisfactory bio assays are those in which a comparison is made of the potency of the unknown or test preparation (U) with that of the standard preparation (S) In hormone estimations as in all other types of assay *the design should be selected with care bad designs* frequently cause great wastage of both time and animals

Gaddum (1953 *a*) has recently reviewed the various experimental designs which have been used in bio assays and his excellent article should be consulted by those interested in this problem In hormone estimations in patients the two most popular types of assay have been those employing a three point

or a four point design[1] Assays depending on a six point design[1] are more reliable than either of the preceding but are too cumbersome for use in the clinical field

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[1] Three point design = two doses of S and one of U
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INDEX OF PRECISION ($\lambda$) —This term was introduced by Gaddum in 1933 and is an estimate of the standard deviation of the logarithms of the individual effective doses (S D log tolerance) In assays depending on graded effects the index is calculated by dividing the standard deviation of the responses (usually termed $s$) by the slope of the line connecting the response with the logarithm of the dose (usually termed $b$), i e, $\lambda = \frac{s}{b}$, in quantal assays the symbol $b$ has a slightly different meaning and $\lambda$ is estimated from the expression $\frac{1}{b}$ The precision of a given assay is high when $s$ is small and $b$ is large From this it follows that the lower the figure for $\lambda$ the more precise the assay and the higher the $\lambda$ the less precise the assay

The great advantage of $\lambda$ lies in the fact that it is independent of such factors as the design of the assay and the number of animals employed For this reason the index serves as a very satisfactory means of comparing the precision of one test with that of another In the opinion of Gaddum (1953 *a*) all statements of errors of tests should include an estimate of $\lambda$ in addition to any other information that may be thought desirable

It is very difficult to make a definite statement as to what degree of precision is acceptable in clinical bio assays It may, however be useful to divide the tests into three groups

(*a*) ASSAYS IN WHICH THE $\lambda$ IS 0 2 OR LESS —Such assays are very precise and are well suited for clinical studies
(*b*) ASSAYS IN WHICH THE $\lambda$ LIES BETWEEN 0 2 AND 0 3 —Such tests are less precise than those in (*a*) but can still be used with reasonable confidence
(*c*) ASSAYS IN WHICH THE $\lambda$ IS 0 3 OR MORE —Such assays have a low degree of precision and are unsuitable for quantitative work

## THE RELATIVE MERITS OF BLOOD AND URINE DETERMINATIONS

This subject has been recently reviewed by Marrian (1955), and the subsequent two paragraphs dealing with steroid hormones are quoted directly from his article

Following the administration of certain steroid hormones to human subjects only small and somewhat variable proportions of the doses administered can be recovered from the urine in the form of identifiable metabolites or in the form of the unchanged hormones Such findings suggest that the amounts of the steroid hormones and their metabolites which are excreted in the urine may represent in these cases only small and variable fractions of the amounts of these hormones which are secreted in the body Because of this some authorities have questioned the value of urinary steroid hormones as a means of assessing the secretory activities of the steroid hormone secreting glands and have urged that blood determinations are to be preferred where suitable methods are available

While the drawbacks of a urinary steroid determination as a means of accurately assessing hormone secretion must be admitted, there would seem to be no justification for regarding a blood determination as a preferable alternative for this purpose A urine determination will yield a value which may be accepted as bearing some approximate proportionality to the total amount of hormones secreted during a certain period of time—usually from eight to twenty four hours On the other hand, a blood determination will provide information about the amount of hormone or its metabolites which are present in the blood at one particular instant of time—the instant when the blood sample is withdrawn This information may, of course be just as valuable as or in some circumstances even more valuable than that provided by a urine determination but it must be emphasised that it is information of a different kind Accordingly λ blood and urine determinations should not be regarded as alternatives whose relative merits can be argued about but rather as sources of different kinds of information which may be supplementary to one another

As will be seen in subsequent chapters methods for the determination of hormones and their metabolites in urine are with a few exceptions more reliable than those for the estimation of the corresponding substances in blood At the time of writing the only hormones which can be estimated with reasonable accuracy in both blood and urine are HCG and some steroids, but only in the case of HCG have reliable

calculations been made of the renal clearance in health and disease (Gastineau *et al*, 1949, Loraine, 1950)

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CHAPTER II

# *Pituitary Gonadotrophins*

## INTRODUCTION

THREE gonadotrophic substances are elaborated by the anterior lobe of the pituitary These are the *follicle stimulating hormone* (FSH) the *interstitial cell stimulating* or *luteinising hormone* (ICSH or LH) and *luteotrophin* The present chapter deal with assay methods for FSH and ICSH, with methods of extraction of these hormones from urine and with their excretion in normal and pathological conditions in man Luteotrophin is considered in Chapter VII while the placental gonadotrophin *human chorionic gonadotrophin* (HCG) is discussed in Chapter III

### Chemical Nature of Pituitary FSH and ICSH

Workers in two independent laboratories have stated that pituitary ICSH has been obtained in the pure state and that the preparation when subjected to electrophoretic ultra centrifuge and solubility tests behaves as a homogeneous protein (Li *et al*, 1940 Shedlovsky *et al* 1940) According to Li *et al* (1940) sheep ICSH has a molecular weight of 40 000 and an iso electric *p*H of 4 6 The hormone is insoluble in 66 per cent acetone, it contains carbohydrate in its molecule and is therefore referred to as a glycoprotein Shedlovsky *et al* (1940) showed that swine ICSH is also a glycoprotein This substance has a molecular weight of 100 000 and an iso electric *p*H of 7 45 Highly purified preparations of both sheep and pig FSH have been made but it has not yet been claimed that the hormones have been isolated in the pure state Sheep FSH is believed to be a glycoprotein with a molecular weight of 70 000 and an iso electric *p*H of 4 5 The properties of pig FSH are very similar For a fuller discussion of the chemical properties of the pituitary gonadotrophins the reader is referred to

publications by Fevold (1937), Li and Evans (1948), Morris (1955) and Loraine (1956)

Little is at present known regarding the chemical nature of the gonadotrophins in human non pregnant urine Most workers have assumed that two gonadotrophic hormones, FSH and ICSH, are excreted and that these substances are similar in their chemical and biological properties to the hormones isolated from animal pituitaries However, in view of the fact that human urinary FSH has not yet been separated from human urinary ICSH, it would appear that the evidence for the existence of two separate gonadotrophic substances in non pregnant urine is by no means conclusive Indeed the suggestion has recently been made (Segaloff *et al*, 1954) that such urine contains a single gonadotrophin with both follicle stimulating and luteinising activities Definite information regarding the number of gonadotrophic hormones excreted by non pregnant subjects must await further work on the chemical nature and biological properties of these substances

## METHODS OF ASSAY OF PITUITARY GONADOTROPHINS IN URINE

It is generally believed that men non pregnant women and children excrete gonadotrophins with both follicle stimulating and luteinising activities and that these hormones are elaborated by the anterior pituitary The urinary excretion is relatively high at and beyond the menopause but is low in children, in men and in women during reproductive life At present no available assay method is sufficiently sensitive to detect FSH and ICSH activity with any regularity in blood and accordingly the great majority of studies have been conducted on urine which has been extracted and concentrated by a variety of procedures The test animals employed have generally been rats or mice which are more sensitive to stimulation by gonadotrophic hormones than are for example, rabbits, guinea pigs or amphibia Ideally, assays should be performed in hypophysectomised animals in order that the endogenous production of gonadotrophins should not interfere with the results The subject of gonadotrophin assay in relation to clinical problems has recently been reviewed (Loraine, 1956, Albert, 1956)

## 1 Human Menopausal Gonadotrophin (HMG) as a Standard Preparation for the Assay of Pituitary Gonadotrophins in Urine

One of the great difficulties in the estimation of pituitary gonadotrophins in urine is the fact that no International Standard preparation is available for the comparative assay of these substances It has therefore been necessary to express results of clinical assays in arbitrary rat' and 'mouse units,' a unit' being defined as that quantity necessary to produce a given effect Arbitrary units employed in intact and hypophysectomised rats have included the doses necessary to cause vaginal cornification to produce histological changes in the ovaries and to cause 50 to 100 per cent increase in weight of the ovaries uterus, seminal vesicles and ventral lobe of the prostate In intact immature mice most investigators have adopted as a unit the quantity of extract producing 100 to 200 per cent increase in uterine weight A mouse hyperæmia unit has also been proposed (Lloyd *et al*, 1949) and defined as the dose necessary to cause hyperæmia in 25 per cent of eight ovaries

Trevan (1927) was among the first to note that the threshold dose of a substance varies enormously even when an animal colony is kept under relatively constant environmental conditions It is now generally recognised that the error of estimations expressed in animal units without reference to a standard preparation may be threefold or more and that results calculated in this way have little quantitative significance

Recent work by Loraine and Brown (1955 1956 *a*) has shown that urinary gonadotrophins from men and from normally menstruating and post menopausal women can be assayed in terms of human menopausal gonadotrophin (HMG) [1] The standard preparation (HMG 20) was prepared by Messrs Organon (Newhouse Scotland) from the urine of menopausal and post menopausal women The kaolin acetone method of extraction was employed (see p 31) and the crude material obtained by this procedure was treated with tricalcium phosphate in order to reduce toxicity The final

[1] This term will be used to designate the gonadotrophic activity in the urine of menopausal and post menopausal women Such urine extracts contain both FSH and ICSH activity The term HMG was introduced at the first meeting of the Gonadotrophin Club in Geneva (August 1953)

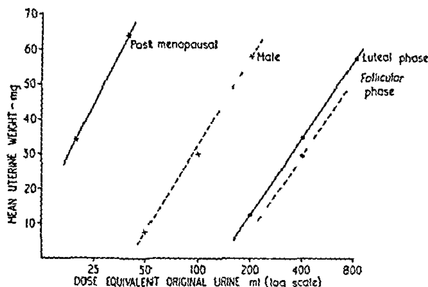



Fig 1

Dose response curves for urinary gonadotrophins from post menopausal women normal men and cyclic women—uterine weight test in intact mice (*From Loraine and Brown* 1956 *a*)

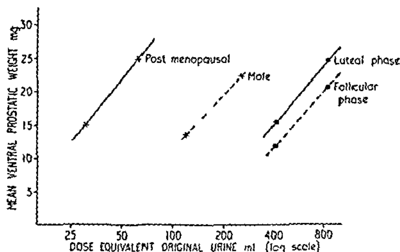



Fig 2

Dose response curves for urinary gonadotrophins from post menopausal women normal men and cyclic women—ventral prostatic weight test in hypophysectomised rats (*From Loraine and Brown* 1956 *a*)

powder was freely soluble in water, it was relatively non toxic and could be administered to intact mice and to hypophysectomised rats up to a total dose of 16 mg Pooled urine was collected from normal male subjects, from normally menstruating women during the follicular and luteal phases of the cycle, and from post menopausal subjects The method of extraction of the gonadotrophins from urine was similar to that adopted for the preparation of the standard

Two bio-assay methods were used, one depending on the enlargement of the uterus in intact immature mice and the other on the enlargement of the ventral lobe of the prostate in hypophysectomised immature rats

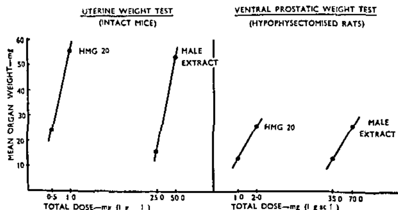



Fig 3
Four point assays of male gonadotrophin against HMG 20
(*From Loraine and Brown* 1956 *a*)

In Figs 1 and 2, which are taken from a paper by Loraine and Brown (1956 *a*) are shown dose response curves for male, post menopausal female and cyclic female gonadotrophins using the mouse uterus and rat prostate tests

It will be noted (Fig 1) that over the dosage range used there was a linear relationship between the log dose and the effect By both tests the post menopausal urine extract was approximately five times more active than pooled male urine, and male urine was approximately four times more active than pooled follicular phase and luteal phase urine

In Figs 3 to 5 are shown typical four point assays in which the various gonadotrophins were compared with HMG 20 using the same two bio assay methods Statistical calculations

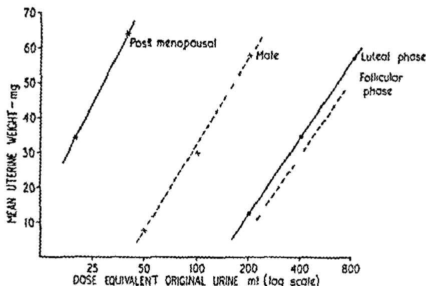



Fig 1

Dose response curves for urinary gonadotrophins from post menopausal women normal men and cyclic women—uterine weight test in intact mice (*From Loraine and Brown* 1956 *a*)

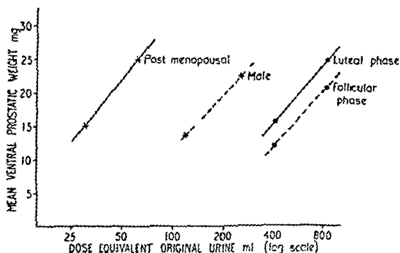



Fig 2

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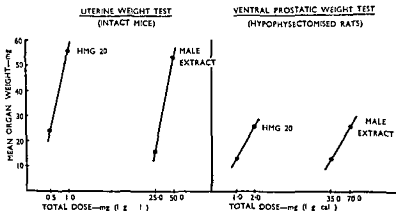



FIG 3

Four point assays of male gonadotrophin against HMG-20
(*From Loraine and Brown* 1956 *a*)

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In Figs 3 to 5 are shown typical four point assays in which the various gonadotrophins were compared with HMG 20 using the same two bio assay methods Statistical calculations

by the techniques recommended by Finney (1952) and by Gaddum (1953 *a*, *b*) demonstrated that there was no *significant difference between the slopes of the various log* dose effect curves Accordingly it is justifiable to conclude

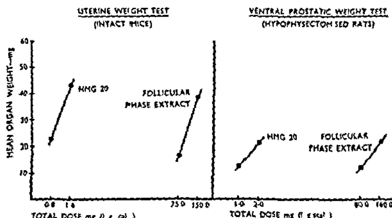



Fig 4
Four point assays of follicular phase gonadotrophin against HMG 20
(*From Loraine and Brown* 1956 *a*)

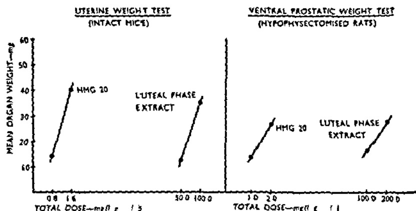



Fig 5
Four point assays of luteal phase gonadotrophin against HMG 20
(*From Loraine and Brown* 1956 *a*)

that urinary gonadotrophins from normal men and from normally menstruating and post menopausal women can be assayed in terms of HMG and that the latter substance can be used as a standard for the assay of all human non pregnant urinary gonadotrophins

In Table I the activity of HMG 20 is compared with that of the other urinary gonadotrophins by both assay methods

With the mouse uterus test the mean ratio of activities (S/U) for male urine was 45, for follicular phase urine 112 and for luteal phase urine 97 The corresponding figures using the rat prostate test were respectively 35 86 and 84 In the assays conducted on post menopausal urine the mean ratios were 4 9 for the mouse uterus test and 3 8 for the rat prostate test It will be seen that there is no significant

TABLE I

COMPARISON OF URINARY GONADOTROPHINS FROM MALES AND FROM NORMALLY MENSTRUATING AND POST MENOPAUSAL WOMEN WITH HMG 20 USING DIFFERENT ASSAY METHODS

(*From Loraine* 1956)

| Urinary Extract Administered | Ratio of Activities Standard/Unknown Uterine Weight Test (Mice) | Ratio of Activities Standard/Unknown Ventral Prostatic Weight Test (Rats) | Mean Index of Discrimination Uterus/Ventral Prostate |
|---|---|---|---|
| Male | 54/1<br>36/1 | 33/1<br>36/1 | 1 30 |
| Follicular phase female | 114/1<br>110/1 | 80/1<br>91/1 | 1 31 |
| Luteal phase female | 125/1<br>68/1 | 87/1<br>80/1 | 1 15 |
| Post menopausal female | 3 0/1<br>6 8/1 | 2 8/1<br>4 8/1 | 1 28 |

difference between the estimations obtained by the two assay methods for the relative potency of the four different gonadotrophic extracts

Gaddum (1955) has recently suggested that data of this type can conveniently be expressed in terms of the *index of discrimination* This is equal to the ratio of the two results which would be obtained if one of the substances were used to assay an unknown extract containing the other In Table I the index of discrimination is shown in column 4 and is obtained by dividing the mean of the ratios in column 2 by the corresponding figure in column 3 It will be noted that with all types of urinary extract the index is approximately equal

to unity This suggests that the active principle measured by the two tests is identical and provides evidence that there is no qualitative difference between the various non pregnant urinary gonadotrophins when these substances are extracted from urine by the kaolin acetone method

## 2 Tests claimed to be Specific for FSH

Most investigators agree that any specific assay method for FSH must employ hypophysectomised animals and that in intact animals the secretion of gonadotrophins from the animal s own pituitary will interfere with the response of the end organs In general, methods have depended on observa tions of the ovaries and testes of rats after hypophysectomy Recently, however, an assay method for FSH has been described using the ovarian weight test in intact immature rats which have been primed with relatively large doses of HCG

Three assay methods for FSH will be considered

(a) Follicular growth in hypophysectomised immature female rats (Evans *et al*, 1939)

(b) Increase in testicular weight in hypophysectomised immature male rats treated with an excess of HCG (Paesi *et al*, 1951)

(c) Increase in ovarian weight in intact immature rats treated with HCG (Steelman and Pohley 1953)

(a) Follicular Growth in Hypophysectomised Immature Female Rats —In the technique described by Evans *et al* (1939) hypophysectomised immature rats were injected sub cutaneously once per day for three days The animals were killed seventy two hours after the first injection and the ovaries examined histologically A unit was arbitrarily defined as the minimum quantity of extract necessary to produce healthy non atretic follicles with small antra This method is claimed to be highly specific for FSH but no information is available about its precision It has the additional advantage that the presence of ICSH in pituitary or urine extracts can be detected by the effect of this hormone on the ovarian interstitial tissue

So far assays by this technique have been mainly restricted to pituitary tissue In the few attempts that have been made to estimate FSH activity in human urine using hypophy sectomised female rats the test has been found to be relatively

insensitive It is therefore necessary to prepare highly concentrated extracts from urine which, in dosages required to produce follicular growth in the ovaries, are frequently toxic to the experimental animals In view of this fact there is little likelihood that this assay method will prove of much value for the estimation of FSH activity in clinical practice

(*b*) Increase in Testicular Weight in Hypophysectomised Immature Rats treated with an Excess of HCG —This test was described by Paesi *et al* (1951) and was used to estimate the FSH content of rat pituitaries Hypophysectomised immature male rats were implanted intra abdominally with pituitary tissue from castrated male and female rats The right testis was removed on the day of implantation All animals received 10 i u of HCG per day for seven days They were killed on the eighth day and the left testis was weighed The difference in weight between right and left testes was taken as an index of the FSH activity of the implanted pituitaries

This assay method was employed by Diczfalusy (1953) to determine whether placental extracts contained demonstrable quantities of FSH but the technique has not so far been employed to assay the FSH content of human urine extracts The method is laborious and tedious and its specificity is somewhat questionable in view of the finding of Simpson *et al* (1944) that both FSH and ICSH are capable of causing testicular growth in hypophysectomised rats

(*c*) Increase in Ovarian Weight in Intact Immature Female Rats treated with HCG —This method depends on the observation that HCG will augment the action of FSH on the rat ovary It has been used by Steelman and Pohley (1953) to assay FSH in pituitary glands Neal *et al* (1954) using rats and Brown (1955) using mice have attempted to adapt the method for studies on human urine In the procedure described by Steelman and Pohley (1953) the animals were injected sub cutaneously thrice per day for three days with swine FSH and with HCG The autopsy was performed seventy two hours after the first injection at which time the ovaries were dissected out and weighed A total dose of 20 i u of HCG per animal was found to produce a satisfactory degree of augmentation of ovarian weight and thereby to increase the sensitivity of the animals to exogenous FSH

This test appears promising as a relatively simple means of

determining FSH activity in human urine Further information should be accumulated regarding its accuracy, precision, specificity and sensitivity

### 3 Tests claimed to be Specific for ICSH

As in the case of FSH most assays for ICSH have been conducted on hypophysectomised animals The following methods will be considered

(*a*) Repair of the interstitial tissue in the ovaries of hypophysectomised immature female rats (Evans *et al*, 1939)

(*b*) Enlargement of the prostate in hypophysectomised immature male rats (Greep *et al*, 1942, McArthur, 1952, Loraine and Brown, 1954)

(*c*) Enlargement of the seminal vesicles in hypophysectomised immature male rats (McArthur, 1952, Loraine and Brown, 1954)

(*d*) Increase in activity of prostatic alkaline phosphatase in hypophysectomised immature male rats (Schaffenburg and McCullagh 1951)

(*a*) Repair of the Interstitial Tissue in Hypophysectomised Immature Female Rats —This test was used by Evans *et al* (1939) to estimate the ICSH content of sheep pituitary extracts The injection schedule was similar to that described for the assay of FSH except that injections were given intraperitoneally instead of subcutaneously ICSH has a specific action in repairing the degenerated interstitial cells resulting from hypophysectomy This is indicated by the reappearance of a normal nuclear pattern and by the disappearance of the characteristic 'wheel cells in the ovaries The test is claimed to be highly specific for ICSH but no information is available about its precision The assay method when applied to the estimation of ICSH in human urinary extracts has been shown to be very insensitive and in view of this fact it is unlikely that the technique will be of any value in the estimation of ICSH activity for clinical purposes

(*b*) Enlargement of the Prostate in Hypophysectomised Immature Male Rats —According to Greep *et al* (1942) this is a unique test in that it is specific for ICSH and is not affected by the presence of FSH The assay method has been used

to estimate ICSH activity in animal pituitaries in male urine and in urine from normally menstruating and post menopausal women (Simpson *et al* 1943, McArthur, 1952, Loraine and Brown, 1954, 1956 *a*) The design of the assay has varied somewhat in different laboratories Greep *et al* (1942) com menced injections on the third day after hypophysectomy, while Loraine and Brown (1954) employed a five day rest period between hypophysectomy and the start of injections Most workers inject the animals subcutaneously once or twice per day for four days and kill them ninety six hours after the first injection In the experience of Loraine and Brown (1954) the precision of the method is greater when the ventral lobe of the prostate, rather than the total gland, is weighed These investigators found that, with the ventral lobe of the prostate as end point, the figure for the index of precision ($\lambda$) was generally less than 0 2, this value would appear to be satisfactory for quantitative work

This assay method is sufficiently sensitive to estimate urinary ICSH activity in normal and pathological conditions in man Although the technique is too tedious and too laborious for routine studies it is probable that valuable information will be obtained by its use in selected patients Recently Segaloff (1955) has stated that prolactin sensitises the prostate to the action of ICSH and that the assay method may not be as specific as was previously believed Segaloff s observation has not been confirmed by Diczfalusy and Loraine (1957)

(*c*) Enlargement of the Seminal Vesicles in Hypophy sectomised Immature Male Rats —This method has been used to estimate ICSH activity in human urine (McArthur, 1952 Loraine and Brown, 1954) The technique of assay is similar to that described for the prostate The test is less sensitive than that depending on prostatic weight and is therefore not to be recommended for the assay of urinary ICSH in man

(*d*) Increase in Activity of the Prostatic Alkaline Phosphatase in Hypophysectomised Immature Male Rats — This method was described by Schaffenburg and McCullagh (1951) and was used by McCullagh *et al* (1953) to estimate urinary ICSH activity in patients with eunuchoidism As described by the authors the method was at best semi quantitative and probably did not satisfy the criteria of a valid bio assay

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## 4 Non specific Assay Methods for Pituitary Gonadotrophins

Such tests generally depend on observations in intact immature rats and mice Assays can be divided according to whether the effect is primary or secondary In the primary group the gonads themselves are inspected while in the secondary group the effects result from the liberation of œstrogens or androgens from the gonads Some of the tests proposed by various investigators are shown in Table II

Many of these assay methods are not sufficiently sensitive to measure gonadotrophic activity in human non pregnant urine and will not be discussed by the author Consideration will be given to the following three methods

(*a*) The mouse uterus test
(*b*) The ovarian hyperæmia test in rats and mice
(*c*) The test depending on the expulsion of spermatozoa in *Xenopus lævis*

(*a*) Mouse Uterus Test —This assay method has gained wide popularity for the routine estimation of pituitary gonado trophins in human urine It is a highly sensitive test and gives positive results with the majority of urine extracts Most centres have used the technique described by Klinefelter *et al* (1943) or some modification thereof Intact immature mice weighing 8 to 10 g are injected subcutaneously once or twice per day for three days and are killed approximately seventy two hours after the first injection Results of clinical assays are expressed in mouse uterine units of FSH per twenty four hours a unit being defined as that quantity necessary to produce a given effect—usually 100 to 150 per cent increase in uterine weight The disadvantages of expressing results in animal units has already been mentioned In addition it should be emphasised that the mouse uterus test measures a mixture of FSH and ICSH activities and should not be regarded as in any way specific for urinary FSH

Few estimates are at present available in the literature regarding the precision of this method In the experience of Loraine and Brown (1954 1956 *a*) the figure for the index of precision (λ) was generally less than 0 2 this would appear to be satisfactory for quantitative work In four point assays in which urinary extracts from males and from non pregnant

TABLE II

METHODS OF ASSAY OF PITUITARY GONADOTROPHINS IN INTACT ANIMALS

(*From Loraine* 1956)

| Primary Group | | | | Secondary Group | | | |
|---|---|---|---|---|---|---|---|
| Animal | Index of Response | Material Administered | Reference | Animal | Index of Response | Material Administered | Reference |
| Immature rat | Ovarian weight | Sheep pituitary extract | Wallen Lawrence and Van Dyke (1931) | Immature rat | Uterine weight | Rat pituitary extracts | Heller *et al* (1938) |
| Immature rat | Formation of corpora lutea | Bovine pituitary extracts | Janssen and Loeser (1930) | Immature rat | Vaginal opening | Ox sheep and swine pituitary extracts | Wallen Lawrence and Van Dyke (1931) |
| Immature rat | Ovarian hyperæmia | Human urine | Farris (1946) | Immature rat | Vaginal cornification | Sheep pituitary extracts | D Amour and D Amour (1938) |
| Immature mouse | Histological changes in ovary | Sheep pituitary extracts | Thomopoulou and Li (1954) | Immature rat | Seminal vesicle weight | Sheep pituitary extracts | Fevold (1939) |
| Immature mouse | Ovarian hyperæmia | Human urine extracts | Lloyd *et al* (1949) | Immature mouse | Uterine weight | Human urine extracts | Levin and Tyndale (1937) |
| Immature mouse | Ovarian weight | Human urine extracts | Levin and Tyndale (1937) | | | | |
| Œstrus rabbit | Ovulation | Ox pituitary extracts | Hill *et al* (1934) | | | | |
| Ring dove | Testicular weight | Ox and dove pituitary extracts | Riddle and Flemion (1928) | | | | |
| *Xenopus laevis* | Expulsion of spermatozoa | Ox pituitary extracts Human urine extracts | Hobson and Landgrebe (1954) | | | | |

(*c*) EXPULSION OF SPERMATOZOA IN XENOPUS LÆVIS — Although amphibia are now widely used in the diagnosis of pregnancy, few studies on non pregnant urine have so far been reported Recently Hobson and Landgrebe (1954), using male *Xenopus lævis*, have demonstrated gonadotrophic activity in an extract of menopausal urine prepared by the kaolin acetone method Further observations with this technique in male and cyclic female urine will be awaited with interest

## EXTRACTION OF GONADOTROPHINS FROM HUMAN NON-PREGNANT URINE

Many methods have been proposed for the preparation of gonadotrophins from human urine The procedures differ considerably in nature and complexity Usually, however, they suffer from the common disadvantages that they are laborious and tedious that considerable loss of gonadotrophic activity may occur during their performance and that the final extracts obtained are often toxic to the experimental animals The following methods will be briefly discussed

1 Alcohol precipitation
2 Acetone precipitation
3 Tannic acid precipitation
4 Benzoic acid adsorption
5 Kaolin adsorption with acetone precipitation
6 Aluminium hydroxide adsorption
7 Permutit adsorption
8 Ultrafiltration

### 1 Alcohol Precipitation

This technique with various modifications has been used by numerous investigators including Zondek (1931), Heller and Heller (1939) and Klinefelter *et al* (1943) The *p*H of the urine is adjusted to 4 to 6 aliquots of urine are precipitated with 4 or 5 volumes of 95 per cent alcohol and are left to stand overnight On the following morning the supernatant fluid is removed the residue centrifuged and the precipitate washed with ether The powder is dried and is then eluted several times with tap water the suspension being centrifuged each time and the supernatant fluid retained Varney and Koch

females were compared with HMG there was no significant difference between the slopes of the various log dose effect curves This finding supports the view that, when assays are conducted by the mouse uterus test, HMG can be used as a standard preparation for all estimations involving non pregnant urine

(*b*) OVARIAN HYPERÆMIA TEST IN RATS AND MICE —This technique in rats has been used both for the diagnosis of pregnancy and for the estimation of urinary and serum HCG throughout normal pregnancy (see Chapter III) Farris (1946) has suggested that the test may also be employed in non pregnant subjects to determine the time of ovulation Unextracted urine was administered subcutaneously to intact immature rats and the ovaries examined for hyperæmia two hours later In normal subjects positive reactions were found on three or four successive days of the cycle at a time at which ovulation generally occurs Other investigators, however, have been unable to confirm Farris's findings and the technique is now little used as a test for ovulation

Lloyd *et al* (1949) claim that the mouse is ten to twenty times more sensitive than the rat and have used the mouse hyperæmia test as an assay method for urinary ICSH in non pregnant individuals Intact immature mice were injected subcutaneously with urine extracts and the ovaries were examined for hyperæmia after an interval of seven hours Hyperæmia producing material was found in relatively large quantities at the time of ovulation Males excreted less hyperæmia producing material' than normal females and the output was also relatively low in castrate women

Tests depending on hyperæmia can be criticised mainly on grounds of specificity Spontaneous reactions are relatively frequent, and the success of the method appears to be largely conditioned by the strain of animal employed It has been shown by Payne (1951) and by Smith (1955) that cortisone and cortisol interfere significantly with the hyperæmia response to HCG a similar interference may well occur in the case of FSH and ICSH Finally, the technique depends on an all or none (quantal) rather than a graded response (see Chapter I) Gaddum (1933) and Perry (1950) have shown that for any given degree of accuracy, quantal assays require approximately twice as many observations as assays depending on graded effects

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(1942) found that the yield of gonadotrophic material was greater with alcohol than with tannic acid precipitation They also showed that crude extracts prepared by alcohol precipitation were less toxic to experimental animals than those prepared by the tannic acid or benzoic acid methods

In general, the alcohol precipitation technique has given satisfactory results in high titre urines such as are found at the menopause and in various types of ovarian and testicular failure However, in low titre urines, *e g*, in males and in normally menstruating women, the crude alcohol extracts are frequently too toxic for routine use Attempts to reduce toxicity, *e g*, by dialysis of the final solution against tap water, may seriously interfere with the yield of gonadotrophins and for this reason are not to be recommended in clinical studies

## 2 Acetone Precipitation

Investigators in the United Kingdom have generally preferred acetone to alcohol as a precipitating agent for reasons of economy (Loraine, 1949, 1950, Dekanski, 1949) The technique of extraction is very similar to that described for the alcohol method In clinical studies satisfactory results are obtained when the excretion of gonadotrophins is high, but in low titre urines extracts prepared by simple acetone precipitation are frequently toxic to the experimental animals Attempts to reduce toxicity *e g*, by dialysis may seriously interfere with the yield of gonadotrophins

## 3 Tannic Acid Precipitation

In the method described by Levin and Tyndale (1936) the *p*H of the urine was adjusted to 5 aliquots of urine were precipitated with an aqueous 10 per cent tannic acid solution and were centrifuged after standing The precipitate which was called the crude tannate powder, was extracted repeatedly with 80 to 95 per cent alcohol and was washed several times with acetone The crude material could be purified by repeated extraction with a barium hydroxide boric acetate mixture at *p*H 9 to 10 followed by acetone precipitation

Some investigators including Pedersen Bjergaard (1936) and Levin (1941) have used extracts prepared by tannic acid precipitation in clinical studie Other workers however (Heller and Heller, 1939, Varney and Koch 1942), were

unable to obtain quantitative yields of gonadotrophin by the tannic acid method and found that extracts prepared in this way were too toxic for routine use In the opinion of the author the tannic acid procedure is more laborious and time consuming than techniques depending on alcohol precipitation and kaolin adsorption For this reason tannic acid precipitation cannot be recommended as a routine procedure for the extraction of gonadotrophins from non pregnant urine

**4 Benzoic Acid Adsorption**

In the procedure described by Katzman and Doisy (1934) the urine was precipitated with acetone saturated with benzoic acid The precipitate was washed with an aqueous solution of benzoic acid and was dissolved in cold acetone The acetone insoluble material containing the gonadotrophins was washed with acetone and dissolved in dilute NaOH Any insoluble material was removed by centrifugation The extract was further concentrated by acetone precipitation the precipitate was dried and was dissolved in the desired volume of water

Various workers including Katzman (1937) and Varney and Koch (1942) have compared the alcohol precipitation and benzoic acid adsorption techniques They found that the yield of gonadotrophins obtained by the benzoic acid method was considerably lower than that by alcohol precipitation It is evident that the benzoic acid procedure cannot be recom mended for the extraction of gonadotrophins from human non pregnant urine when quantitative assays are required The method is however simple and rapid and is well adapted to industrial conditions where it may be necessary to prepare large quantities of active material

**5 Kaolin Adsorption with Acetone Precipitation**

In 1941, Scott described a method for the extraction of HCG from pregnancy urine The hormone was adsorbed on kaolin at *p*H 4 and eluted by 0 1 N NaOH Extracts prepared by the original kaolin method are not however sufficiently concentrated to permit the estimation of gonadotrophins in non pregnant urine Accordingly various workers including Bradbury *et al* (1949) Loraine (1949 1950) and Dekanski (1949) have after adsorption and elution, precipitated the gonadotrophins by acetone or alcohol Extracts prepared by

the kaolin acetone or kaolin alcohol techniques have proved suitable for administration to intact mice or hypophysectomised rats (McArthur, 1952, Loraine and Brown, 1954, 1956 *a*)

The kaolin acetone extraction method has recently been reinvestigated by a series of recovery experiments in which HMG and HCG were added to pooled male urine (Loraine

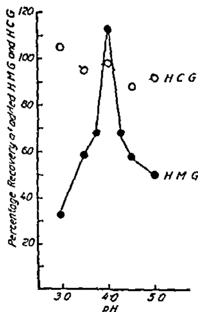



Fig 6

Relationship between the *p*H of adsorption on kaolin and the recovery of HMG and HCG added to male urine *p*H of elution 11 to 11 5 (*From Loraine and Brown* 1954)

and Brown, 1954) Urine extracts were assayed biologically by the mouse uterus test The results are shown in Figs 6 and 7

It will be noted that in the case of HMG, accurate control of the *p*H was essential at both adsorption and elution stages of the procedure The critical *p*H for adsorption is 4, when the *p*H falls to 3 5 or rises to 4 5 approximately 40 per cent of the material is not adsorbed (Fig 6) For elution of HMG a *p*H of 11 to 11 5 is critical, losses of approximately 50 per cent are encountered at *p*H s of 10 and 12 (Fig 7) These results indicate that figures obtained by the kaolin acetone method

for the excretion of pituitary gonadotrophins in normal and pathological conditions have little quantitative significance unless the optimal *p*H's for adsorption and elution have been employed It is apparent from Figs 6 and 7 that, for HCG the *p*H range for maximal adsorption and elution is wider Accordingly when this hormone is extracted from pregnancy urine by the kaolin method the *p*H s need not be controlled so accurately

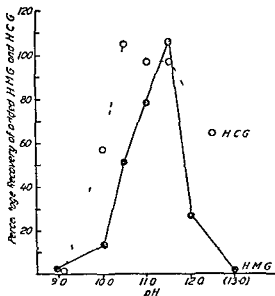



Fig 7

Relationship between the *p*H of elution from kaolin and the recovery of HMG and HCG added to male urine *p*H of adsorption 3 9 to 4 (*From Loraine and Brown* 1954)

In subsequent studies Loraine and Brown (1956 *a*) extracted gonadotrophins from pooled male urine and from urine collected during the follicular and luteal phases of the normal menstrual cycle A similar series of recovery experiments were undertaken in which these extracts were added to pooled male urine and their biological activity determined using the mouse uterus test at different stages of the kaolin acetone procedure The importance of accurate control of the *p*H was again emphasised, and it was shown that the optimal *p*H s for adsorption and elution of male and cyclic female gonadotrophins were very similar to those for HMG

the kaolin acetone or kaolin alcohol techniques have proved suitable for administration to intact mice or hypophysectomised rats (McArthur, 1952, Loraine and Brown, 1954, 1956 *a*)

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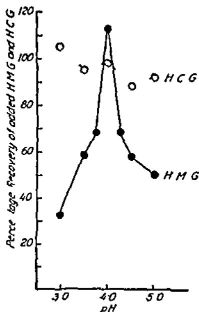



Fig 6

Relationship between the *p*H of adsorption on kaolin and the recovery of HMG and HCG added to male urine *p*H of elution 11 to 11·5 (*From Loraine and Brown* 1954)

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It will be noted that, in the case of HMG, accurate control of the *p*H was essential at both adsorption and elution stages of the procedure The critical *p*H for adsorption is 4, when the *p*H falls to 3·5 or rises to 4·5 approximately 40 per cent of the material is not adsorbed (Fig 6) For elution of HMG a *p*H of 11 to 11·5 is critical, losses of approximately 50 per cent are encountered at *p*H s of 10 and 12 (Fig 7) These results indicate that figures obtained by the kaolin acetone method

**7 Permutit Adsorption**

Katzman *et al* (1943) described a chromatographic procedure for the preparation of HCG from pregnancy urine in which the hormone was adsorbed on to permutit and was eluted by ammonium acetate in alcohol Johnsen (1955 *a b*) has slightly modified the method and has applied it to the extraction of gonadotrophins from pooled post menopausal urine The method is both simple and rapid and yields highly active extracts of low toxicity *In its present form the technique is* not suitable for the quantitative extraction of gonadotrophins from twenty four hour urine specimens but may be of value for the large scale preparation of active material

**8 Ultrafiltration**

Gorbman (1945) described a method in which the urinary gonadotrophins were ultrafiltered through a collodion membrane under pressure When filtration was completed the membrane along with the protein hormone residue which had not passed through the pores of the membrane, was removed from the filter and placed in an alcohol ether solution in a centrifuge tube The alcohol ether dissolved the collodion membrane and precipitated the gonadotrophins The solution was then centrifuged the supernatant fluid discarded and the residue washed repeatedly with alcohol and with ether Before bio-assay the dry precipitate was taken up in water or saline Van Gibe (1955) has recently investigated the role of adsorption in the ultrafiltration procedure and has estimated that 80 to 90 per cent of the gonadotrophic activity is adsorbed on to the collodion membrane

Jungck *et al* (1947) compared the relative merits of the ultrafiltration and alcohol precipitation dialysis methods They concluded that the former technique was preferable in that it was less complex less laborious and less expensive

## POSSIBILITIES OF SEPARATING THE DIFFERENT GONADOTROPHIC FACTORS BY TRICALCIUM PHOSPHATE

**1 Separation of HCG from HMG**

Crooke Butt and their collaborators were the first to fractionate crude kaolin acetone extracts of urine on tricalcium

Kaolin acetone powders from low titre urines are sometimes toxic to experimental animals, especially hypophysectomised rats, since they contain relatively large quantities of inactive substances which are sparingly soluble in water Further purification of these extracts is therefore necessary and such a procedure has recently been described (Loraine and Brown, 1956 *a*) This involves elution of the crude kaolin acetone powders with water under controlled conditions and treatment of the eluate with tricalcium phosphate These purification steps do not result in any significant loss of gonadotrophic activity and make it possible to administer the equivalent of approximately one litre of original urine to an intact mouse or a hypophysectomised rat

In the opinion of the author the kaolin acetone method with accurate *p*H control is the procedure of choice for the routine extraction of gonadotrophins from human non pregnant urine In low titre urines purification of the crude material with tricalcium phosphate may be necessary The technique is less tedious and laborious than the majority of published methods and gives better yields of gonadotrophins than are obtained by tannic acid precipitation and benzoic acid adsorption In addition, extracts prepared in this way are generally less toxic to experimental animals than those prepared by simple alcohol or acetone precipitation

## 6 Aluminium Hydroxide Adsorption

This technique which has been described by Malburg and Goodman (1954) is similar in many respects to the kaolin acetone method The urinary gonadotrophins were adsorbed on 10 per cent aluminium hydroxide at *p*H 4 and were eluted by normal NaOH at *p*H 10 The *p*H of the eluant was adjusted to 6 and the hormones precipitated by acetone The precipitate was separated by centrifugation washed with ether and taken up in water

Malburg and Goodman (1954) found this technique both simple and rapid the yields of gonadotrophins were almost identical with those obtained by the alcohol precipitation dialysis technique This procedure appears worthy of a trial as a routine method for the extraction of gonadotrophins from non pregnant urine

It will be noted that the adsorption of the gonadotrophins on tricalcium phosphate was influenced markedly by the $pH$ of the solutions HMG was not adsorbed to any appreciable extent on tricalcium phosphate at $pH$ s above 6 and HCG was adsorbed completely below $pH$ 7 The adsorption of HCG on tricalcium phosphate decreased as the $pH$ increased and no adsorption occurred at $pH$ 10 and above These results indicated that the separation of HMG from HCG was possible by differential adsorption on tricalcium phosphate

It is possible that this separation technique might prove of value in testing for the presence of pituitary gonadotrophins in pregnancy urine and in assessing whether the pituitary contributes significantly to the very high gonadotrophin titre in human urine in the first trimester of normal pregnancy and to the abnormally high excretion of gonadotrophin found in some patients with pre eclamptic toxæmia and pregnancy complicated by diabetes Various workers including Hasenbein (1952) and Lyon *et al* (1953), have claimed that human pregnancy urine contains FSH activity This interesting observation requires confirmation

**2 Separation of Human Urinary FSH from Human Urinary ICSH**

In their earlier communications Crooke and his co workers suggested that the tricalcium phosphate technique might be a means of separating human urinary FSH from human urinary ICSH This suggestion was not borne out by the findings of Loraine and Brown (1954 1956 *a*) who prepared crude kaolin acetone powders from post menopausal urine from male urine and from follicular phase and luteal phase female urine and treated the crude extracts with tricalcium phosphate These workers found that all the extracts prepared in this way contained relatively large amounts of ICSH activity as estimated by the ventral prostatic weight test in hypophysectomised immature rats A typical dose effect curve for HMG is shown in Fig 9

In view of these observations it appears reasonable to conclude that treatment of urine extracts with tricalcium phosphate does not effect any separation of human urinary FSH from human urinary ICSH

As emphasised previously the tricalcium phosphate

phosphate columns (Crooke and Butt 1952, Crooke *et al*, 1954) These workers eluted the gonadotrophins from the columns by disodium hydrogen phosphate ($Na_2HPO_4$) and by trisodium phosphate ($Na_3PO_4$) The material eluted by disodium hydrogen phosphate was referred to as Gonadotrophin A (GA) and that eluted by trisodium phosphate as Gonadotrophin B (GB) [1] Assays of the eluates by biological methods showed

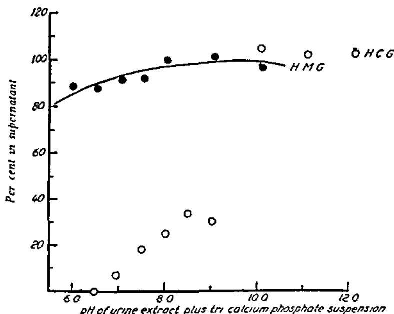



Fig 8

Relationship between the *p*H and the adsorption of HMG and HCG on tricalcium phosphate (*From Loraine and Brown* 1954)

that GA was excreted predominantly at the menopause and at certain times during the normal menstrual cycle while GB was the main gonadotrophin in the urine of pregnant women

Loraine and Brown (1954) have recently studied the tricalcium phosphate procedure as a means of separating HMG from HCG For the assay of HCG the test depending on the enlargement of the prostate in intact immature rats was used HMG activity was estimated by the mouse uterus test The results are shown in Fig 8

[1] In the opinion of the author the terms GA and GB are unfortunate in that they may be confused with prolan A and prolan B to which they bear no relationship

It will be noted that the adsorption of the gonadotrophins *on tricalcium phosphate was influenced markedly* by the *p*H of the solutions HMG was not adsorbed to any appreciable extent on tricalcium phosphate at *p*H s above 6 and HCG was adsorbed completely below *p*H 7 The adsorption of HCG on tricalcium phosphate decreased as the *p*H increased and no adsorption occurred at *p*H 10 and above These results indicated that the separation of HMG from HCG was possible by differential adsorption on tricalcium phosphate

It is possible that this separation technique might prove of value in testing for the presence of pituitary gonadotrophins in pregnancy urine and in assessing whether the pituitary contributes significantly to the very high gonadotrophin titre in human urine in the first trimester of normal pregnancy and to the abnormally high excretion of gonadotrophin found in some patients with pre eclamptic toxæmia and pregnancy complicated by diabetes Various workers, including Hasenbein (1952) and Lyon *et al* (1953), have claimed that human pregnancy urine contains FSH activity This interesting observation requires confirmation

**2 Separation of Human Urinary FSH from Human Urinary ICSH**

In their earlier communications Crooke and his co workers suggested that the tricalcium phosphate technique might be a means of separating human urinary FSH from human urinary ICSH This suggestion was not borne out by the findings of Loraine and Brown (1954 1956 *a*) who prepared crude kaolin acetone powders from post menopausal urine from male urine and from follicular phase and luteal phase female urine and treated the crude extracts with tricalcium phosphate These workers found that all the extracts prepared in this way contained relatively large amounts of ICSH activity as estimated by the ventral prostatic weight test in hypophysectomised immature rats A typical dose effect curve for HMG is shown in Fig 9

In view of these observations it appears reasonable to conclude that treatment of urine extracts with tricalcium phosphate does not effect any separation of human urinary FSH from human urinary ICSH

As emphasised previously the *tricalcium phosphate*

purification step is of considerable value in reducing the toxicity of crude kaolin acetone extracts By incorporating this step in

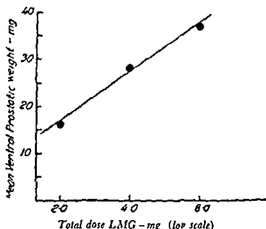



Fig 9

Relationship between the log dose of HMG and the ventral prostatic weight in hypophysectomised immature rats—weight of ventral prostate in uninjected animals = 8 mg (*From Loraine and Brown* 1954)

the extraction procedure it is possible to administer to intact mice and to hypophysectomised rats total doses equivalent to approximately one litre of original urine

## CHEMICAL ESTIMATION OF URINARY GONADOTROPHINS

Many investigators have emphasised the desirability of estimating urinary gonadotrophins by chemical means in order to avoid laborious and time consuming bio assay procedures, and with this end in view various attempts have been made to find some chemical property which consistently parallels biological activity For example Bowman (1941) studied the oxidation reduction characteristics of HCG He found that the hormone became biologically inactive when treated with oxidising agents and that activity could be restored by reducing agents, it was suggested that the reducing capacity of HCG might be used as the basis of a chemical test for the diagnosis of pregnancy Maxwell and Bischoff (1935) showed that reagents known to react with amino groups caused partial or

complete inhibition of the biological activity of pituitary gonadotrophins Various workers, including Li (1949), have stated that the hexose content of the gonadotrophin molecule is important in relation to biological activity Stran and Jones (1954) and Crooke *et al* (1954) have attempted to estimate gonadotrophins by testing urinary extracts for proteins and sugars but there is at present no evidence to support the view that the excretion of these groups of substances in the urine does in any way reflect the output of gonadotrophins as measured by bio assay

Until chemical methods of assay become more specific they cannot replace biological techniques in the estimation of urinary gonadotrophins for clinical purposes

## A QUANTITATIVE METHOD FOR THE ROUTINE DETERMINATION OF GONADOTROPHINS IN HUMAN NON PREGNANT URINE

Such a technique has recently been developed by Loraine and Brown (1956 *a b*) The main steps are as follows

1 Extraction of the urine by the kaolin acetone method with accurate control of the *p*H at all stages of the procedure
2 In low titre urines treatment of the crude kaolin acetone powders with tricalcium phosphate in order to reduce the toxicity of the extracts
3 Bio assay by the mouse uterus test
4 Expression of results in terms of human menopausal gonadotrophin (HMG) as HMG units per twenty four hours At the second meeting of the Gonadotrophin Club (Birmingham England, 1955) it was suggested that the Organon preparation HMG 20A might be adopted as a provisional standard and that one unit should be defined as the activity contained in 1 mg of HMG 20A

The technique is sufficiently sensitive to measure the gonadotrophin excretion in normal and pathological conditions in man The end point of the bio assay is not specific for either FSH or ICSH and gives a measure of what may be conveniently termed total gonadotrophic activity In the

purification step is of considerable value in reducing the toxicity of crude kaolin acetone extracts By incorporating this step in

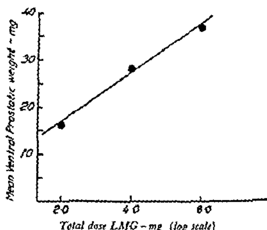



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It would be of considerable interest to study the gonadotrophin excretion before and during puberty using the more reliable methods of extraction and bio assay now available

**2 Normally Menstruating Women**

The gonadotrophin excretion during the normal menstrual cycle has been studied by numerous investigators including Smith and Smith (1936) Klinefelter *et al* (1943) and Pedersen Bjergaard and Tonnesen (1948) It is generally stated that the output is low in the follicular and luteal phases and that a peak of excretion occurs at or about the time of ovulation D Amour (1943) attempted to determine the time of ovulation by a variety of tests including basal temperature records vaginal smears and the excretion of œstrogens pregnanediol and gonadotrophins He claimed that the gonadotrophin peak was the most reliable index that ovulation had occurred This view has been challenged by Heller *et al* (1944) who found that the pattern of gonadotrophin excretion was very variable from one patient to another and concluded that the stage of the ovarian cycle and the time of ovulation could not be predicted by estimating the output of urinary gonadotrophins A similar conclusion was reached by Main *et al* (1943) who reported the presence of two gonadotrophin peaks in a proportion of their cases

It is evident that little is at present known regarding the normal pattern of excretion of gonadotrophins during the menstrual cycle Much further work with improved methods of extraction and bio assay is required before the range of normal is definitely established Single random estimates of gonadotrophin output are of little value and may even be misleading, they should be replaced by serial determinations in individual cases

On the basis of assays involving histological changes in the ovaries of hypophysectomised immature rats it has been stated that female urine is predominantly follicle stimulating in nature (Bahn *et al* 1953) More recent work by McArthur (1952) and by Loraine and Brown (1956 *a*) using the prostate test in hypophysectomised rats has demonstrated that normal female urine at all stages of the cycle contains significant amounts of ICSH activity

experience of the authors the bio assay method is relatively precise and has an index of precision (λ) which is generally below 0 2 In experiments in which HMG was added to pooled male and cyclic female urine and extracted and assayed as described above, the recoveries for the whole method varied from 52 to 92 per cent with a mean figure of 76 per cent (Loraine 1957 *a*) This figure was considered to be reasonably satisfactory considering the many steps involved in the extraction procedure and the additional inherent error of the bio-assay

## THE EXCRETION OF GONADOTROPHINS IN NORMAL NON PREGNANT SUBJECTS

Existing methods of estimation of pituitary gonadotrophins for clinical purposes are very unsatisfactory and take the form of qualitative tests rather than quantitative assays Most workers express results in 'animal units'—usually 'mouse uterine units'—and for this reason it is not possible to compare results from different laboratories Accordingly at the time of writing it would be misleading to quote normal figures in units for the excretion of gonadotrophins in non pregnant subjects The introduction of a standard preparation such as HMG 20A should increase considerably the reliability of such determinations and should make it possible to compare directly results from different centres

### 1 Children

All investigators agree that the gonadotrophic activity in the urine of children and adolescents is very low One of the most detailed studies in the literature is that of Nathanson *et al* (1941) These workers extracted gonadotrophins from urine by alcohol precipitation and their bio assay end point depended on the production of follicular stimulation in the ovaries of intact immature mice In boys no activity was detected before the age of thirteen, but in girls positive results were generally obtained at the age of eleven It was noted that in girls activity was often detected approximately one year before the onset of menstruation In a study of the gonadotrophin excretion in young and adolescent boys Catchpole *et al* (1938) concluded that the titre was related to developmental status rather than to chronological age

in those in whom the menopause occurred more than twenty five years before the investigation The mean output in the latter group however, was still considerably higher than that found in women during reproductive life

Much experimental work has been carried out on the biological properties of menopausal and post menopausal gonadotrophins In intact and hypophysectomised rats these hormones are potent stimulators of follicular growth without much effect on the interstitial tissue and this has led to the assumption that the material is predominantly, if not entirely, follicle stimulating hormone (FSH) Borth *et al* (1954) and Loraine and Brown (1954) using the prostate test in hypophysectomised rats have recently demonstrated that HMG is, in addition a relatively rich source of ICSH activity

## THE EXCRETION OF PITUITARY GONADOTROPHINS IN PATHOLOGICAL CONDITIONS

### 1 Disorders of Menstruation

At the present time no reliable information is available in *the literature regarding the gonadotrophin excretion in patients* with menstrual abnormalities In the few studies which have been undertaken the methods of extraction and bio-assay employed have been far from satisfactory and it is debatable whether the results reported are of any value whatsoever from the quantitative point of view It must be emphasised that many difficulties have been encountered by investigators who have attempted to assay urinary gonadotrophins in these patients The chief of these has been the lack of a method sufficiently sensitive to detect activity in such low titre urines while another formidable obstacle to clinical progress has been the necessity for conducting serial determinations instead of single random estimations in individual cases

In view of these problems it is not surprising that until now urinary gonadotrophin assays have proved of little value to the gynæcologist in the differential diagnosis of patients with various types of menstrual abnormalities However the outlook for the future is not unpromising During recent years methods for the determination of pituitary gonadotrophins in urine have become more sensitive and more precise (see p 39) and as a consequence it should soon be possible to obtain

### 3 Normal Men

Gonadotrophic activity has been detected in male urine by numerous investigators including Leathem and Levin (1941) and Klinefelter *et al* (1943) The amount excreted is generally somewhat higher than that in normally menstruating women during the follicular and luteal phases of the cycle

Few reports are available in the literature regarding the excretion of gonadotrophins in men at different ages, but there is no evidence to suggest that the output rises significantly with advancing years The most careful study is probably that of Schou (1951) who found that the output remained relatively constant in normal men between the ages of thirty nine and sixty three This worker concluded that in the male there was no equivalent of the female menopause at which time the excretion of gonadotrophins generally rises steeply

Male gonadotrophins resemble female gonadotrophins in their biological properties, and most workers have stated that they are predominantly follicle stimulating in nature (Fraenkel Conrat *et al*, 1940) More recent work by Loraine and Brown (1956 *a*) has shown that a relatively large amount of ICSH activity is also present

### 4 Post menopausal Women

All investigators agree that the gonadotrophin titre in blood and urine is greatly elevated at and beyond the menopause This is believed to represent an attempt on the part of the pituitary to stimulate unresponsive ovaries a similar effect occurs after castration in men and women The relationship between the gonadotrophin excretion and the development and severity of menopausal symptoms will be discussed later in this chapter

Few studies have so far been reported of the gonadotrophin excretion in post menopausal subjects One of the more detailed investigations is that of Heller and Shipley (1951) These workers were unable to show any correlation between the gonadotrophin output on the one hand and the chronological age of the patient on the other They did, however, find that a relationship existed between the amount excreted and the duration of time since the onset of the menopause In patients who were less than twenty five years beyond the menopause the mean excretion was significantly higher than

of this group which occurs relatively frequently in clinical practice In this disease the main symptom is continuous bleeding which may last for many weeks ovulation does not occur and the endometrium shows cystic hyperplasia without any evidence of secretory activity The ætiology of the condition is not known with certainty It has, however, been suggested that, in metropathia there may be an abnormally low secretion of pituitary FSH and ICSH and that this deficiency may result in failure of ovulation and lack of formation of the corpus luteum Swyer (1950) has put forward the alternative view that in this disease the mode of secretion of the pituitary gonadotrophins may be different from that in normal individuals He suggests that in normally menstruating women FSH and ICSH are produced in a cyclic fashion while in patients with metropathia there is a relatively constant secretion of FSH which eventually causes bleeding from a proliferative endometrium It must be emphasised that neither of these hypotheses has as yet been substantiated by hormone assay findings in blood and urine The suggestions are of interest, but in the present state of our knowledge they should be accepted with some reserve

There have been few reports on the gonadotrophin excretion in patients with the various forms of dysfunctional uterine hæmorrhage The field is one which would repay careful study The most extensive investigation at present available is probably that of Pedersen Bjergaard and Tønnesen (1951) who determined the urinary excretion of gonadotrophins in 222 patients with various types of menstrual anomaly other than amenorrhœa Great variations in output in individual cases were noted but the majority of the readings lay within the normal range Unfortunately the precision of the bio assay method employed by these investigators was probably not high and it is doubtful if much quantitative significance can be attached to the results they obtained

(*c*) DYSMENORRHŒA —Gonadotrophin assays by reliable methods have not yet been reported in patients with this condition

## 2 The Menopausal Syndrome

There is a great body of evidence in the literature to support the view that at the menopause, the urinary excretion and

reliable and useful information by assays conducted on low titre as well as on high titre urines Accordingly, it can be stated with some confidence that, with the passage of time, gonadotrophin assays will play an increasingly important role in gynæcological practice The information obtained from such assays will almost certainly be of considerable clinical value in relation to the diagnosis, prognosis and treatment of patients with gynæcological disorders

(*a*) AMENORRHŒA —Gonadotrophin assays in such patients may be expected to provide information which is of value in differentiating amenorrhœa resulting from pituitary failure from amenorrhœa due to ovarian deficiency In pituitary amenorrhœa the excretion of pituitary gonadotrophins is generally very low and often no activity can be detected even by the mouse uterus test, on the other hand ovarian amenorrhœa is usually associated with a greatly increased output of urinary gonadotrophins

Fluhmann (1929) found gonadotrophic activity in the blood of six out of seventeen patients with amenorrhœa In the ten patients studied by Frank *et al* (1937) four showed abnormally high blood levels and in six no activity could be detected *Pedersen Bjergaard and Tønnesen* (1951) estimated the urinary excretion of pituitary gonadotrophins in 110 patients with primary amenorrhœa and in 221 cases of secondary amenorrhœa These workers extracted the hormones from urine by tannic acid precipitation and conducted bio assays using the mouse uterus test They found that in the patients with primary amenorrhœa 35 per cent had abnormally high readings, and that in 65 per cent the excretion was within or below the normal range In cases of secondary amenorrhœa the corresponding figures were 34 and 66 per cent

It is not known whether, in amenorrhœic subjects the gonadotrophin output remains relatively constant over long periods of time or shows evidence of cyclical variations Serial determinations in such patients might well give information of clinical importance

(*b*) DYSFUNCTIONAL UTERINE HÆMORRHAGE —This is a general term used to denote all forms of abnormal menstrual bleeding for which no organic cause can be found The subject is discussed more fully in Chapter IX

The condition known as *metropathia hæmorrhagica* is a member

(*d*) Menopausal symptoms have occasionally been observed in patients with gonadal failure secondary to pituitary failure In cases of this type the urinary excretion of gonadotrophins is abnormally low

The biological properties of menopausal and post menopausal gonadotrophins have already been discussed (see p 43) It is probable that at the menopause, both FSH and ICSH are excreted in relatively large quantities

## 3 Ovarian Deficiency

It has already been emphasised that the excretion of pituitary gonadotrophins in urine is abnormally high in the presence of ovarian failure such as occurs at the menopause or after irradiation of the ovaries Ovarian deficiency is however probably best exemplified by the rare condition known variously as ovarian agenesis, ovarian dwarfism or *Turner's syndrome* Since the original description of this disease by Turner (1938) the clinical features and hormone excretion in such patients have been studied by numerous investigators In classical cases the main clinical features include sexual infantilism, short stature, diminished pubic and axillary hair, rudimentary or absent ovaries amenorrhœa and various congenital defects such as webbing of the neck ocular lesions, mental retardation congenital deafness coarctation of the aorta cubitus valgus pes cavus and spina bifida Polani *et al* (1954) have recently demonstrated that the skin of patients with Turner s syndrome has a chromosomal pattern which is characteristically seen in males These workers have suggested that in fact such patients are genetic males

Hormone studies have generally shown (*a*) diminished output of urinary œstrogens (*b*) diminished output of urinary 17 ketosteroids (*c*) abnormally high levels of urinary gonado trophins In some patients studied the urinary gonadotrophin titre has been in the same range as that encountered in menopausal and post menopausal subjects

Little or no information is at present available on the bio logical nature of the gonadotrophins excreted in Turner's syndrome since most assays have been made with the mouse uterus test which is not specific either for FSH or for ICSH activity Studies in hypophysectomised male and female rats will be awaited with interest

serum concentration of pituitary gonadotrophins are greatly increased As early as 1929 Fluhmann, using the Aschheim Zondek test, demonstrated that the serum of menopausal and oophorectomised subjects contained relatively large amounts of gonadotrophic activity A similar conclusion was reached by Zondek (1930) who conducted assays on urine extracts rather than blood

Numerous theories have been advanced with respect to the relationship of hormone secretion to menopausal symptoms, particularly flushings One of these suggests that the symptoms result from a decrease in the levels of circulating œstrogens This theory is discussed in Chapter IX Another view is that the symptoms are caused directly by the over production of pituitary gonadotrophins The evidence for and against this theory of gonadotrophin excess will now be briefly considered

According to Albright (1936) there is a definite correlation between the gonadotrophin titre on the one hand and the severity of symptoms on the other This worker using rather unreliable assay methods, claimed that in patients with very high gonadotrophin levels in blood and urine hot flushes were more severe and occurred more frequently This finding has not been confirmed by other investigators and there is now much evidence to support the view that high gonadotrophin levels play little or no part in the genesis of menopausal symptoms The main objections to the ' theory of gonado trophin excess have been reviewed by Fluhmann (1944) and are as follows

(a) High gonadotrophin readings occur in menopausal and post menopausal women in the absence of symptoms The titre remains high long after menopausal symptoms have disappeared

(b) Various conditions which usually show high gonado trophin levels in blood and urine are not associated with menopausal symptoms These conditions include Turner s syndrome, Klinefelter s syndrome and pre pubertal gonadal failure

(c) Improvement of menopausal symptoms can be produced by small doses of œstrogens by sedatives or by pituitary irradiation without affecting the gonadotrophin levels in blood and urine

groups were termed respectively eunuchoidism with low FSH eunuchoidism with normal FSH and eunuchoidism with high FSH This classification is rather unsatisfactory in view of the fact that the bio assay method used by these investigators (the mouse uterus test) is not specific for urinary FSH

Nelson (1953) divides cases of testicular deficiency with the clinical features of eunuchoidism into two groups, depending on the gonadotrophin excretion These groups he terms respectively hypogonadotrophic eunuchoidism and hyper gonadotrophic eunuchoidism In a study of 148 patients Heller and Nelson (1948) reported that 62 per cent of their cases showed an abnormally low excretion of pituitary gonadotrophins while the remaining 38 per cent showed raised excretion figures

Two special syndromes associated with testicular failure merit special consideration These are the conditions which have been termed respectively *Klinefelter s syndrome* and the *male climacteric*

KLINEFELTER S SYNDROME —In 1942 Klinefelter *et al* described a group of patients in whom the presenting features were gynæcomastia small testes and sterility in association with varying degrees of impairment of androgenic function Testicular biopsy showed hyalinisation of the seminiferous tubules with destruction of the Sertoli cells apparently normal Leydig cells and azoospermia The condition which is rela tively rare occurs at puberty or during adolescence, the male climacteric supervenes in all cases by the age of twenty five Hormone assay studies showed that the urinary excretion of 17 ketosteroids was within or slightly below the normal range and that the output of pituitary gonadotrophins as measured by the mouse uterus test was greatly increased The cause of the disease is unknown and none of the theories suggested for the endocrine abnormalities in these patients is entirely satisfactory A similar syndrome without gynæcomastia has been reported by Heller and Nelson (1945) while del Castillo *et al* (1947) have described a group of patients in whom absence of spermatogenesis without impairment of Leydig cell function was associated with normal levels of urinary gonadotrophins

No information is at present available on the biological nature of the gonadotrophins excreted in Klinefelter s syndrome,

### 4 Ovarian Tumours

Gonadotrophin assays by reliable methods have not yet been reported in patients with these neoplasms Greene (1950) states that in cases of feminising ovarian tumour, *e g*, granulosa cell tumour, the excretion of pituitary gonadotrophins is very low and that after removal of the neoplasm the urinary titre rises Hain (1949) conducted hormone excretion studies on a child with a dysgerminoma of the ovary, the only abnormality found was an increased output of urinary gonadotrophin as indicated by a strongly positive Aschheim Zondek test

It is obvious that much further work is required before any conclusions can be drawn regarding the clinical value of gonadotrophin assays in patients with ovarian tumours The field is one which might repay careful investigation

### 5 Testicular Deficiency

Pituitary gonadotrophin assays in patients with testicular deficiency can be used to differentiate primary testicular failure from testicular failure secondary to lesions of the pituitary In the latter group of patients the output of urinary gonadotrophins is usually very low, while in the former abnormally large quantities are generally excreted Nelson (1948), Howard *et al* (1950) and others have stated that this subdivision into primary and secondary gonadal failure may be of importance from the therapeutic point of view Cases of secondary gonadal failure might in theory at least, be expected to respond favourably to treatment with such preparations of gonadotrophins as are at present available, cases of primary gonadal failure, on the other hand, already show abnormally high gonadotrophin levels in blood and urine and should probably be treated *ab initio* by androgens For further information on the various aspects of testicular failure the reader is referred to a recent review by Bishop (1954)

Albright and his collaborators in Boston, and Nelson and his associates in Iowa have studied the urinary excretion of pituitary gonadotrophins in a large number of patients with various types of testicular failure In 144 cases observed by the Albright group (Howard *et al*, 1950) the gonadotrophin excretion was below normal in 26 per cent, normal in 28 per cent and abnormally high in 46 per cent These three

## 7 Acromegaly

There are few reports in the literature regarding the excretion of pituitary gonadotrophins in patients with this condition Klinefelter *et al* (1943) conducted assays in ten such patients and found the excretion to be very variable in that normal, low and high readings were found It is reasonable to assume that the amount of gonadotrophin excreted depended greatly on the stage in the evolution of the disease at which the determination was made In early cases, when the clinical features are those of hyperpituitarism high urinary readings are to be expected On the other hand, in the later stages of the condition, when symptoms of hypopituitarism supervene the excretion will tend to be below the normal range

## 8 Panhypopituitarism

All investigators agree that in this condition the urinary excretion of gonadotrophins is abnormally low Usually no activity can be detected even when highly concentrated urine extracts are assayed by relatively sensitive biological methods such as the mouse uterus test The largest series of cases reported is probably that of Klinefelter *et al* (1943) who in fourteen patients found that the mean excretion of urinary gonadotrophins was much lower than that obtained in normal men or in normally menstruating women of a comparable age group Results similar to those of Klinefelter *et al* (1943) were obtained by Easterling *et al* (1951) It has been rightly emphasised by many workers that the diagnosis of panhypopituitarism should be made only if no gonadotrophic activity is detected in the urine by the mouse uterus test If such activity is present the diagnosis should be carefully reviewed and probably discarded

## 9 Mammary Carcinoma

Segaloff *et al* (1954) have claimed that the gonadotrophin excretion may be of prognostic significance in patients suffering from mammary carcinoma These workers have stated that if prior to treatment the urinary gonadotrophin output is below the range normally expected for the age of the patient the response to various forms of therapy tends to be unsatisfactory It must be emphasised that the assay methods used

since most assays have been made using the mouse uterus test Such information can only be obtained by the use of hypophysectomised animals

THE MALE CLIMACTERIC —In this condition the functional activity of the Leydig cells is impaired and the production of testicular androgen diminished The main clinical features are hot flushes, impotence, loss of libido, weakness and excessive fatigue Numerous investigators including Howard *et al* (1950) have claimed that in cases of this syndrome the urinary excretion of pituitary gonadotrophins is abnormally high

## 6 Testicular Tumours

Careful and detailed studies of the gonadotrophin excretion in over 400 patients with testicular tumours have been made by Hamburger and his co workers in Copenhagen (Hamburger and Godtfredsen 1941 , Hamburger, 1946) The bio assay methods employed by these investigators to differentiate the various types of gonadotrophins depended on histological changes in the ovaries of intact immature mice and on the increase in ovarian weight in intact immature rats The following account is based on Hamburger s observations

Certain malignant tumours, *e g*, chorionepithelioma and related tumours classified as mixed epitheliomas, contain syncytial trophoblastic like cells and were shown to excrete human chorionic gonadotrophin (HCG) Gonadotrophin assays in patients with this type of tumour are discussed in Chapter III Cases of seminoma, on the other hand, excreted abnormally large quantities of gonadotrophins which were qualitatively indistinguishable from those excreted by meno pausal and post menopausal subjects The excretion of these hormones did not cease after removal of the tumour and bore no relationship to the presence or absence of metastases In addition gonadotrophic activity could not be detected either in extracts of the seminoma itself or in metastatic deposits from the primary tumour In view of these findings it was concluded that the anterior pituitary and not the tumour tissue was responsible for the elaboration of the hormones The cause of the increased excretion of pituitary gonadotrophins in cases of seminoma is not yet known Hamburger (1946) has suggested that this effect may be secondary to the decreased production of androgenic hormones by the testis

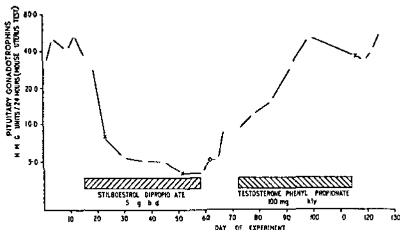



Fig 10

The effect of stilbœstrol and testosterone on the urinary excretion of gonadotrophins in a post menopausal subject with mammary carcinoma (Mrs M F *æt* 62) The open circles denote that the reading was actually less than the figure quoted (*From Loraine* 1957 *c*)

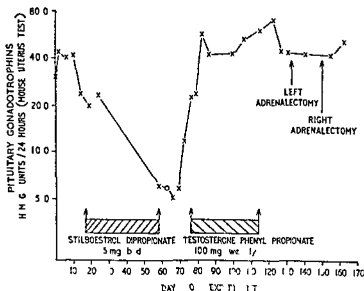



Fig 11

The effect of stilbœstrol testosterone and bilateral adrenalectomy on the urinary excretion of gonadotrophins in a post menopausal subject with mammary carcinoma (Miss J D *æt* 57) The open circle denotes that the reading was actually less than the figure quoted (*From Loraine* 1957 *a*)

by Segaloff *et al* (1954) are open to criticism, and for this reason their claims must be accepted with some reserve

In a recent study in Edinburgh the gonadotrophin excretion has been estimated in approximately fifty post menopausal subjects with mammary carcinoma before treatment with stilbœstrol (Loraine, 1957 *b*, Loraine *et al*, 1957) After a course of therapy lasting six weeks the patients were classified into three groups depending on their response, these groups were designated (*a*) worse' (*b*) no apparent change' and (*c*) improved It was found that in patients classified as worse the mean gonadotrophin excretion before treatment was significantly higher than that obtained in patients classified either as no apparent change' or improved A comparison was also made of the gonadotrophin output in patients with mammary carcinoma with that of a group of subjects of similar age suffering from diseases other than cancer It was found that the mean excretion value in the worse group was significantly higher than that in the control series, while in the groups designated no apparent change and 'improved' such a difference was not demonstrated The reason for the abnormally high gonadotrophin output in the group of patients classified as 'worse' is obscure The problem is one for future elucidation

## THE EFFECTS OF VARIOUS FORMS OF TREATMENT ON THE URINARY EXCRETION OF PITUITARY GONADOTROPHINS

### 1 Œstrogens

It is well established that œstrogens, if administered to patients in sufficiently large dosage will inhibit the secretion of pituitary gonadotrophins and thereby cause a decreased urinary excretion of these hormones Typical responses are shown in Figs 10 and 11 (Loraine, 1957 *a*, *c*) which demonstrate the effect of relatively large dosages of oral stilbœstrol on the gonadotrophin excretion in two post menopausal subjects suffering from mammary carcinoma Bio assays were conducted using the mouse uterus test and results were expressed in terms of HMG-20A

It will be noted that stilbœstrol produced a rapid fall in the gonadotrophin output and that this fall persisted as long

other hand Sohval and Soffer (1951), in a more detailed study involving twenty two patients, found that both ACTH and cortisone produced an increase in the gonadotrophin excretion in approximately 50 per cent of cases

Now that more reliable methods are available for the quantitative determination of gonadotrophins in non pregnant urine, this subject should be reinvestigated

**5 Thyroid Hormone**

Statland and Lerman (1950) reported that in post menopausal subjects with myxœdema treatment with thyroid extract produced a rise in the gonadotrophin excretion This observation was not confirmed by Beierwaltes and Bishop (1954), who could demonstrate no consistent change in gonadotrophin levels in a comparable group of subjects so treated It is probable that these divergent findings can be explained on a methodological basis

**6 Castration**

It is well known that the gonadotrophin excretion rises after both oophorectomy and orchidectomy In Fig 12

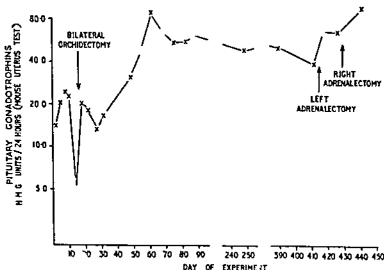



Fig 12

The effect of bilateral orchidectomy and bilateral adrenalectomy on the urinary excretion of gonadotrophins in a male patient with mammary carcinoma (Mr J C *æt* 70) (*From Loraine* 1957 *a*)

as the drug was being administered After cessation of therapy the readings rose steadily and within approximately one month had reached levels in the same range as those in the pre treatment period

It has been suggested (Tokuyama *et al*, 1954) that in post menopausal patients the potency of various natural and synthetic œstrogens may be compared by their ability to depress the urinary excretion of pituitary gonadotrophins This interesting suggestion requires further study

**2 Androgens**

Many investigators, including Heller and Nelson (1945) and McCullagh and Hruby (1949) have demonstrated that androgens are relatively weak inhibitors of pituitary gonadotrophin secretion This observation is borne out by the results presented in Figs 10 and 11 which show the effect of testosterone as well as stilbœstrol on the gonadotrophin excretion in two post menopausal subjects with mammary carcinoma It will be noted that a dosage of testosterone propionate of 100 mg per week intramuscularly did not appear to inhibit the urinary excretion of pituitary gonadotrophins During this period of treatment the output rose to levels in the same range as those encountered prior to the administration of stilbœstrol

**3 Adrenalectomy**

Little information is at present available on the effect of adrenalectomy on gonadotrophin excretion In the female patient shown in Fig 11 bilateral adrenalectomy did not affect the output of gonadotrophins while in the male subject shown in Fig 12 the operation produced a slight rise in excretion

**4 ACTH and Cortisone**

The effect of these hormones on the gonadotrophin excretion has not yet been adequately studied and such evidence as is at present available in the literature is conflicting Mason *et al* (1948) and Sprague *et al* (1950) conducted assays in a very small number of subjects before and after therapy with ACTH and cortisone and stated that these hormones had no demonstrable effect on the gonadotrophin output On the

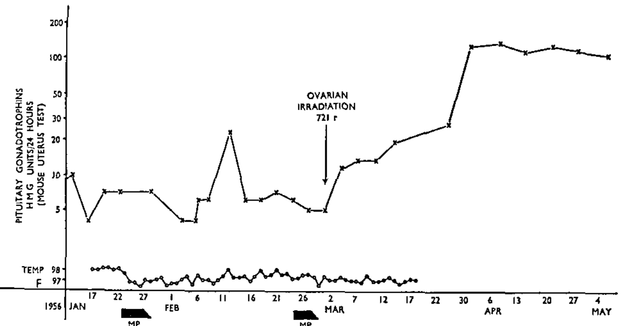



Fig 13

The effect of ovarian irradiation on the urinary excretion of gonadotrophins in a pre menopausal subject with mammary carcinoma (Mrs A R *aet* 41) M P = menstrual period (*From Loraine* 1957 *a*)

(Loraine, 1957 *a*) is shown the effect of bilateral orchidectomy on the gonadotrophin output in a male patient with mammary carcinoma

It will be noted that the level began to rise approximately one month after the operation and that after two months a threefold increase in excretion values had occurred over those in the pre operative period

### 7 Ovarian Irradiation

The effect on gonadotrophin excretion is similar to that produced by oophorectomy A typical example in a pre menopausal patient with mammary carcinoma is shown in Fig 13

In this subject the excretion was studied first during a normal menstrual cycle and secondly subsequent to ovarian irradiation It will be noted that readings in the follicular and luteal phases of the cycle were very low and that a peak of excretion occurred at approximately mid cycle Immediately after ovarian irradiation the excretion values rose steeply and approximately one month after radiotherapy were some twenty times higher than those found in the follicular and luteal phases of the menstrual cycle

### 8 Hypophysectomy

Within recent years hypophysectomy has been practised as a treatment for various types of advanced carcinoma If a complete hypophysectomy has been performed it is reasonable to expect that gonadotrophic activity will disappear from the urine, and it is therefore probable that gonadotrophin assays will be of value to the surgeon as a means of assessing the completeness of the operation Such assays should be particularly informative in menopausal and post menopausal women in whom the pre operative excretion of the hormones will usually be high

## CLINICAL STUDIES INVOLVING PARALLEL ASSAYS OF URINARY GONADOTROPHINS

In Figs 14 and 15 (Loraine, 1957 *c*) are presented the results obtained when urinary gonadotrophin estimations are performed in the same patients using two different assay

Fig 14 shows the gonadotrophin excretion in a pre menopausal subject with mammary carcinoma but without any previous history of menstrual abnormalities It will be noted that the results obtained by the two assay procedures at *all stages of the cycle agree very closely* *In the early follicular* phase the gonadotrophin excretion was low being usually less than 10 units per twenty four hours In the late follicular

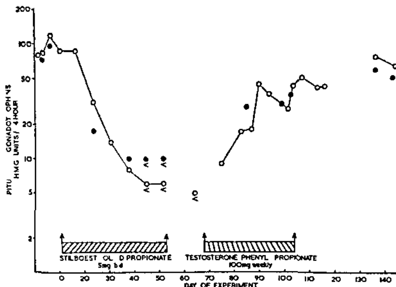



Fig 15

The effect of stilbœstrol and testosterone on the urinary excretion of gonadotrophins in a post menopausal subject with mammary carcinoma (Mrs M S *at* 61) Open circles denote assays conducted by the mouse uterus test closed circles denote assays conducted by the ventral prostat c weight test The sign < indicates that the reading was actually less than the figure quoted (*From Loraine* 1957 *c*)

phase the levels started to rise and reached a maximum at about the sixteenth day of the cycle This maximum is probably associated with the occurrence of ovulation and may be termed the ovulation peak At this time readings by the two methods lay between 25 and 30 units per twenty four hours Subsequent to *ovulation the excretion values decreased rapidly, and in the* luteal phase and at the time of menstruation were again less than 10 units per twenty four hours

Fig 15 shows the effect of œstrogens and androgens on the urinary excretion of gonadotrophins as estimated by the same

methods The methods employed were the uterine weight test in intact mice and the ventral prostatic weight test in hypophysectomised rats The former method is relatively non specific and probably measures a mixture of FSH and

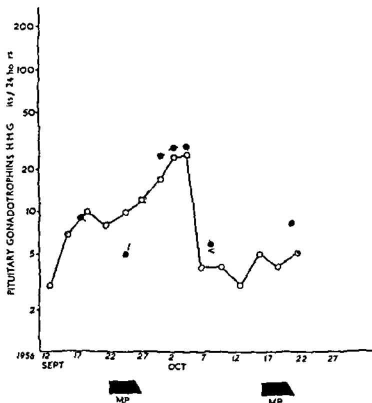



Fig 14

Urinary excretion of gonadotrophins in a pre menopausal subject with mammary carcinoma (Mrs W N *aet* 47) M P = menstrual period Open circles denote assays conducted by the mouse uterus test closed circles denote assays conducted by the ventral prostatic weight test The sign < indicates that the reading was actually less than the figure quoted (*From Loraine* 1957 *c*)

ICSH activity, the latter is generally believed to be specific for ICSH activity The urine samples were extracted by the kaolin acetone method (see p 31) and, in extracts in which gonadotrophic activity was expected to be low the crude kaolin acetone powders were treated with tricalcium phosphate in order to reduce toxicity Results of both assays were expressed in terms of HMG 20A as HMG units per twenty four hours

bio assay method of choice This test is not specific for either FSH or ICSH and gives a measure of total gonadotrophic activity Results of the assay should be expressed in terms of HMG as units per twenty four hours The prostatic weight test in hypophysectomised immature *male rats is sufficiently* sensitive to estimate ICSH activity in clinical conditions but is probably too tedious and laborious for routine studies

The kaolin acetone method with accurate *p*H control is a suitable procedure for the extraction of gonadotrophins from human non pregnant urine Treatment of the crude kaolin acetone powders with tricalcium phosphate makes it possible to administer without toxic effects the equivalent of one litre of original urine to intact mice and hypophysectomised rats

Chemical methods of assay of gonadotrophins are relatively non specific and should not at present replace biological techniques

Both FSH and ICSH activity are present in post menopausal urine in normal male urine, and in urine from normally menstruating women It is not yet clear whether the effects are due to two separate substances or to a single substance with both activities

In normal individuals the excretion of pituitary gonado trophins is low in children in men and in women during reproductive life In menopausal and post menopausal women, on the other hand high excretion values are found

The main indication for pituitary gonadotrophin assays in the clinical field is in the differentiation of primary gonadal failure from gonadal failure secondary to lesions of the pituitary In addition assays may sometimes be helpful in certain types of testicular tumour and in various gynæcological conditions It is probable that in the future gonadotrophin determinations will be of value to the surgeon as a means of assessing the completeness of a hypophysectomy

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two assay methods The patient studied was a post menopausal subject with mammary carcinoma It will be noted that prior to œstrogen therapy the readings obtained by both methods were relatively high, lying in the range 70 to 120 units per twenty four hours The oral administration of stilbœstrol in a dosage of 5 mg twice daily produced a prompt fall in the urinary gonadotrophin output This decrease, which persisted throughout the period of therapy, was of similar magnitude with both the mouse uterus and rat prostate tests Within some five weeks of commencing œstrogen therapy the amounts excreted were so small that no activity could be detected by either test even when relatively large dose equivalents of the original urine were administered Subsequent therapy by testosterone phenyl propionate in a dosage of 100 mg weekly by the intramuscular route did not appear to inhibit the secretion of pituitary gonadotrophins During this time the excretion values obtained by both methods rose in a *corresponding manner and at the end of the period of study* lay in the range 40 to 60 units per twenty four hours

The results presented above indicate that when gonado trophins are extracted from human non pregnant urine by the kaolin acetone method and are assayed by the mouse uterus and rat prostate tests the index of discrimination (uterus/prostate) as defined on page 21 generally approximates unity It might have been anticipated that varying proportions of urinary FSH to urinary ICSH might have been reflected in differences in this index The finding of an index in the neighbourhood of unity suggests either that the material prepared by the kaolin acetone method contains a single gonadotrophin with two activities or that it contains two gonadotrophins which are usually present in the same relative proportions

## Summary and Conclusions

Urinary gonadotrophins from normal men and from normally menstruating and post menopausal women can be assayed in terms of human menopausal gonadotrophin (HMG) It is proposed that a preparation of HMG be used as a standard for the assay of human non pregnant urinary gonadotrophins

For the routine determination of pituitary gonadotrophin excretion in clinical practice the mouse uterus test is the

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## CHAPTER III

# *Human Chorionic Gonadotrophin*

## INTRODUCTION

IN 1927 Aschheim and Zondek demonstrated that the blood and urine of pregnant women contained a gonad stimulating substance which, when injected subcutaneously into intact immature female mice, produced follicular maturation, luteinisation and hæmorrhage into the ovarian stroma. Aschheim and Zondek believed that this gonadotrophic substance was produced by the anterior pituitary but subsequent work by other investigators proved beyond all doubt that the placenta and not the pituitary was responsible for the elaboration of the hormone. Probably the most conclusive evidence in favour of the placental origin of HCG comes from the work of Jones *et al* (1943) and of Stewart *et al* (1948) who showed that the gonadotrophin was produced *in vitro* in placental tissue culture and that this property was exhibited by the chorionic villi being especially marked in the Langhans cells.

The chorionic hormone should be termed *chorionic gonadotrophin* (*CG*) or *human chorionic gonadotrophin* (*HCG*). Names such as "pregnancy prolan" or "anterior pituitary like substance" (APL) are of historical interest only and should no longer be used. HCG and pituitary ICSH are different chemical substances but resemble one another in their biological properties. Both hormones are predominantly luteinising in action.

### 1 Chemical Nature of HCG

HCG is a glycoprotein with a relatively high carbohydrate content in the form of galactose. Gurin *et al* (1940) stated that its molecular weight was 100 000. Purified preparations of HCG have been made by Gurin *et al* (1940), by Lundgren *et al* (1942) and by others but it is doubtful if the hormone

has yet been isolated in the pure state The most active preparations of HCG so far described are those of Gurin et al (1940) and of Katzman et al (1943) The potency of these preparations ranged from 6,000 to 8,000 i u per mg

**2 The International Standard for HCG**

This was established in 1939 under the auspices of the League of Nations By definition 1 i u is the activity contained in 0 1 mg of the standard preparation All assay results should now be expressed in international units, and in this way results from different laboratories can be compared directly There is no longer any excuse for measuring HCG activity in animal units, and it is regrettable that publications still appear in which rat and mouse units are employed

## METHODS OF ASSAY OF HCG

Assay methods for HCG are very numerous The subject has been reviewed by Emmens (1939), Thayer (1946), Loraine (1952, 1954, 1956 a) and Diczfalusy (1953, 1954) For quantitative determinations of HCG in blood and urine, intact immature rats and mice have been favoured as test animals but for pregnancy diagnosis rats, mice rabbits and amphibia have been widely used As in the case of pituitary gonadotrophins it is possible to classify the tests according to whether the effect is *primary* or *secondary* Tables III and IV, which are largely based on data calculated by Diczfalusy (1954), show some of the methods used and compare their relative precision in terms of Gaddum s $\lambda$

**1 Assays depending on Primary Effects**

Many of these methods have considerable disadvantages when applied to the quantitative assay of HCG in the blood and urine of human subjects The method based on the formation of *corpora lutea* in the rat ovary is relatively sensitive, it has, however a very large error and is little better than a qualitative test This was the technique used by Smith and Smith (1934 1936 1941) in studies of the serum concentration of HCG in pre eclamptic toxæmia and also

by White (1947, 1949) in similar estimations in pregnant diabetics. *Ovarian weight in rats* is now little used as an assay method because it lacks precision and is insensitive. Using this technique Evans *et al.* (1937) constructed one of the earliest curves for the urinary excretion of HCG during normal pregnancy. Methods depending on *ovulation in rabbits* and in *toads* have generally proved reliable as pregnancy tests but are too insensitive to be of great value in quantitative assays.

Within the last five years the two methods in this group which have been most extensively employed in the clinical

TABLE III

PRECISION OF ASSAY METHODS FOR HCG—
PRIMARY EFFECTS

*(From Loraine 1956 a)*

| Index of Response | Index of Precision λ | Reference |
|---|---|---|
| Ovarian weight (rat) | 0.139 | Sealy and Sondern (1940) |
| Ovarian weight (rat) | 0.230 | Diczfalusy and Loraine (1955) |
| Ovarian hyperæmia (rat) | 0.450 | Albert and Berkson (1951) |
| Ovarian hyperæmia (rat) | 0.123 | Borth *et al.* (1957) |
| Expulsion of spermatozoa (toad) | 0.10 to 0.176 | Wohlzogen (1953) |

field are those depending on (*a*) *ovarian hyperæmia in rats* and (*b*) *expulsion of spermatozoa in amphibia*.

(*a*) OVARIAN HYPERÆMIA IN RATS.—The ovarian hyperæmia test was introduced by Zondek *et al.* (1945) for the diagnosis of pregnancy and Albert and his co-workers (Albert 1948, Albert and Berkson 1951) have used the technique for the quantitative determination of HCG in blood and urine. The method is sensitive but may have a low degree of precision as indicated by a figure for the index of precision (λ) of 0.45 (Table III). The chief advantage of the ovarian hyperæmia test lies in the fact that the total time required for the assay is only four hours; this is in marked contrast to most other procedures involving multiple injections

into rats and necessitating seventy two-hour to ninety six hour injection periods The main disadvantages of the test are as follows

(i) The success of the assay appears to depend greatly on the strain of animal employed and in certain strains spontaneous reactions are relatively frequent In a number of laboratories, including that of the author, the technique could not be used as a quantitative assay method for HCG

(ii) The assay depends on a quantal (all or none) rather than on a graded response In a quantal assay the effect on each individual animal is not measured, and the result depends on the percentage of animals which show a definite reaction at a given dose level For any given degree of precision quantal assays require approximately twice the number of animals required by assays depending on graded effects This subject is discussed in Chapter I

(iii) The specificity of the test is questionable in view of the finding that cortisone and cortisol will affect the response of the ovary to injected HCG (Payne, 1951, Smith, 1955) It is possible that blood and urine contain other substances which may also interfere with the reaction

(*b*) EXPULSION OF SPERMATOZOA IN AMPHIBIA —In 1947 Galli Mainini described a new pregnancy test depending on spermiation in the male toad *Bufo arenarum* Since then it has become evident that several other male amphibia can be used for pregnancy diagnosis Recently attempts have been made to adapt the method for the quantitative assay of HCG in blood and urine The amphibia used for this purpose have included *Rana pipiens* (Haskins and Sherman 1952) *Bufo Bufo* (Thorborg and Hansen 1951), *Xenopus lævis* (Hobson 1952) and *Bufo viridis* (Wohlzogen, 1953)

The main advantages of the test are as follows

(i) The method is both simple and rapid, quantitative estimations of HCG potency can be obtained in twenty four hours or earlier

(ii) In the hands of most investigators the test has been reasonably precise (Table III)

(iii) The same animal can be used repeatedly. Most authors state that 1 week's rest is sufficient after a positive response.

The chief disadvantages of the assay method include the following:

(i) Amphibia are relatively insensitive. This may necessitate concentration of urine extracts and the use of large quantities of the standard preparation in order to elicit a positive response.

(ii) The response of the animals to HCG is subject to marked seasonal variation. It is usually stated (Thorborg, 1950) that the sensitivity is at a maximum late in winter and in early spring and is at a minimum in the autumn.

(iii) In individual estimations relatively large numbers of animals are required because the assay depends on a quantal rather than on a graded response.

**2 Assays depending on Secondary Effects**

In this group the majority of assays have been performed using intact immature rats. The following indices of response will be considered:

(*a*) Uterine weight
(*b*) Vaginal smears
(*c*) Prostatic weight
(*d*) Seminal vesicle weight
(*e*) Weight of the total accessory reproductive organs

(*a*) UTERINE WEIGHT.—According to Emmens (1939) this test is sensitive but has a low degree of precision. The technique has been used by Jones *et al.* (1944) to estimate the serum concentration of HCG during normal pregnancy and by Dorfman and Rubin (1947) for studies on pregnancy urine. Diczfalusy and Loraine (1955) found the test reasonably precise but showed that the effect of HCG on the uterine weight in rats was increased approximately twofold when the hormone was dissolved in serum or in plasma instead of saline. In view of this fact these workers concluded that the method was unsuitable for HCG assays conducted on serum or plasma. When the rat uterus test is employed for estimations in pregnancy urine the latter must first be precipitated with alcohol

or acetone in order to remove the œstrogenic hormones These substances, if present in the material to be injected, would interfere significantly with the response and would probably cause an overestimate of HCG potency

(*b*) VAGINAL SMEARS —This method was used by Browne and Venning (1936) and by Venning (1948) to study the urinary excretion of HCG during normal pregnancy, and was employed by Bruner (1951, to determine the distribution of

TABLE IV

PRECISION OF ASSAY METHODS FOR HCG—SECONDARY EFFECTS

(*From Loraine* 1956 *a*)

| Index of Response | Index of Precision λ | Reference |
|---|---|---|
| Uterine weight (rat) | 0 329 | Delfs (1941) |
| Uterine weight (rat) | 0 149 | Dorfman and Rubin (1947) |
| Uterine weight (rat) | 0 240 | Sealy and Sondern (1940) |
| Vaginal smears (rat) | 0 276 | Loraine (1950 *a*) |
| Vaginal smears (vitamin B-deficient rat) | 0 115 | Heard and Winton (1939) |
| Total prostatic weight (rat) | 0 139 | Loraine (1950 *a*) |
| Total prostatic weight (rat) | 0 100 | Loraine (1956 *b*) |
| Ventral prostatic weight (hypophysectomised rat) | 0 150 | Diczfalusy *et al* (1950) |
| Seminal vesicle weight (rat) | 0 200 | Watts and Adair (1943) |
| Seminal vesicle weight (rat) | 0 195 | Loraine (1950 *a*) |
| Seminal vesicle weight (hypophysectomised rat) | 0 495 | Diczfalusy *et al* (1950) |
| Total accessory reproductive organs (rat) | 0 210 | Diczfalusy (1953) |
| Total accessory reproductive organs (rat) | 0 105 | Diczfalusy (1954) |

the hormone in the mother and fœtus at different stages of normal pregnancy The main disadvantage of the method lies in the fact that it depends on a quantal rather than a graded response Loraine (1950 *a*) found the vaginal smear test to be less precise than that depending on prostatic weight (Table IV) According to Heard and Winton (1939) the error of the method can be reduced by using adult aneurine deficient rats which are probably more uniform in response than are intact immature animals

For studies on pregnancy urine the vaginal smear test

cannot be used unless the œstrogens are removed, e g, by acetone or alcohol precipitation prior to bio assay

(c) Prostatic Weight—This method (Loraine, 1950 a) has been shown to be suitable for the quantitative assay of HCG in urine serum and placentæ of patients In the technique used in Edinburgh intact immature rats are injected sub cutaneously once per day on three consecutive days at *approximately twenty four hour intervals They are killed on* the fourth day, at which time the prostates are dissected out and are fixed for twenty four hours On the fifth day the organs are freed from fat, dried and weighed

The test is reasonably precise, the index of precision ($\lambda$) being usually below 0 15 (Table IV) The œstrogens in pregnancy urine do not interfere with the response and for this reason unextracted urine can be injected The effect of HCG on the prostatic weight of intact or hypophysectomised rats is not increased when the hormone is dissolved in serum or plasma instead of saline and accordingly the technique can be used to assay HCG in untreated serum or plasma (Diczfalusy and Loraine, 1955)

(d) Seminal Vesicle Weight—The sensitivity of this test is comparable to that of (c), but the method is less precise (Table IV) The method has been used by Watts and Adair (1943) to estimate the urinary excretion of HCG in cases of pre eclamptic toxæmia and essential hypertension in pregnancy As in the case of (c) the œstrogens in pregnancy urine do not affect the response and for this reason untreated urine can be injected

(e) Weight of Total Accessory Reproductive Organs (total prostate plus seminal vesicles)—Diczfalusy (1954) found that in his strain of animals this test was somewhat more precise than that depending on prostatic weight alone (Table IV) This worker used the method to estimate the placental concentration of HCG at different stages of normal pregnancy

## PREGNANCY DIAGNOSIS TESTS

In early pregnancy very large quantities of HCG are elaborated by the placenta and most biological tests for pregnancy depend on the demonstration of this hormone in body fluids usually in urine A great number of such tests

have been proposed using different animal species and reliability of the various procedures has been thoroughly investigated in the course of the last twenty five years The methods which have been most widely used are as follows

### 1 The Aschheim-Zondek Test

This was the first test to be developed and it depends on the fact that HCG causes the development of hæmorrhagic follicles and corpora lutea in the ovaries of intact immature mice The method gives a reliable diagnosis of pregnancy in the vast majority of cases Its chief disadvantage lies in the fact that five days are required for its performance

### 2 The Friedman Test

In this method rabbits are used The urine is injected intravenously and the end point of the assay is the production of ovulation The result can be obtained within twenty four to forty eight hours The reliability of the procedure is comparable with that of the Aschheim Zondek test

### 3 The Kupperman Test

This method has as its end point ovarian hyperæmia in rats In the original method described by Kupperman *et al* (1943) the ovaries were examined two hours after the intra peritoneal injection of unextracted urine This test is reasonably reliable and has the great advantage of rapidity of performance The original method or some modification thereof is in routine use in many centres

### 4 The Hogben Test

This method depends on the ability of HCG to cause ovulation in the South African clawed toad *Xenopus lævis* The test is reliable and relatively rapid (positive responses are obtained within twenty four hours) The test animals can be used repeatedly The chief disadvantage is the fact that *Xenopus* is rather insensitive to stimulation with HCG, and for this reason it is usually necessary to concentrate the urine prior to administration

### 5 The Galli Mainini Test

HCG will cause expulsion of spermatozoa in male amphibia and this has been made the basis of a pregnancy test which is

now in routine use in many laboratories Tests depending on male amphibia are generally accurate and very rapid results can usually be read within two hours As in the case of female amphibia the same animal can be used repeatedly

In his original experiments Galli Mainini (1947) employed *Bufo arenarum* Since then it has been demonstrated that many other species of amphibia are suitable for pregnancy diagnosis tests For example successful results have been reported with *Rana pipiens* (Haskins and Sherman, 1949), *Bufo Bufo* (Thorborg 1950), *Bufo marinus* (Wannan 1952) and *Xenopus lævis* (Hobson, 1952)

For fuller information on the subject of pregnancy diagnosis the reader is referred to articles by Robbins and Parker (1948) Cowie (1948) Thorborg (1950) and Hobson (1955)

## EXTRACTION OF HCG FROM URINE

The methods used are very similar to those which have been described in Chapter II for pituitary gonadotrophins and include

1 Alcohol precipitation
2 Acetone precipitation
3 Benzoic acid adsorption
4 Tungstic acid precipitation
5 Kaolin adsorption with or without acetone precipitation
6 Permutit adsorption

The end point of the bio assay largely determines whether or not extraction methods for HCG should be employed With relatively insensitive techniques *e g* those depending on male or female amphibia it is often necessary to prepare concentrated extracts in order to elicit a biological response With more sensitive techniques *e g* those employing ovarian hyperæmia or prostatic weight concentration of the urine is not required Assays depending on uterine weight and vaginal smears are relatively sensitive but will not yield valid results unless the œstrogens are removed from pregnancy urine by alcohol or acetone precipitation prior to bio assay As mentioned previously the great advantage of the assay method depending on the prostatic weight in rats, lies in the fact that untreated urine

can be injected as the œstrogens do not interfere with the response of the end organs to HCG

## HCG ASSAYS IN HUMAN SERUM

Many investigators have estimated the serum concentration of HCG in normal and abnormal pregnancy, and in the course of these studies many different bio assay techniques have been employed Recently it has become apparent that in such estimations on untreated serum the end point of the test must be selected with care in order that valid results are obtained Maddock and his collaborators reported that the effect of HCG on the rat uterus was increased fivefold to tenfold when the hormone was administered in human plasma instead of in saline (Maddock and Leach, 1952, Maddock *et al*, 1953, Leach *et al* 1954) In this series of papers however, no attempt was made to calculate the error of the assays and it is doubtful if the results have much quantitative significance Diczfalusy and Loraine (1955) also found that serum and plasma increased the effect of HCG on the rat uterus but reported only an approximate doubling of potency From these results it is apparent however, that bio assays of HCG in serum and plasma by the uterine weight method as recommended by Jones *et al* (1944) and by Dorfman and Rubin (1947) will most probably give an overestimate of the real potency and will thus yield erroneously high serum levels

Diczfalusy and Loraine (1955) have shown that the effect of HCG on the weight of the accessory reproductive organs of immature male rats is not influenced appreciably when the hormone is dissolved in human serum or plasma instead of saline These workers concluded that for clinical bio assays on human serum satisfactory results from the quantitative point of view can be obtained if the prostatic weight seminal vesicle weight and weight of the total accessory reproductive organs are used as indices of response

## THE CLINICAL APPLICATION OF HCG ASSAYS

### 1 Normal Pregnancy

Several investigators have estimated the urinary excretion and serum concentration of HCG throughout normal pregnancy but only in the papers of Jones *et al* (1944), Wilson

*et al* (1949) Loraine (1950 *a*) Albert and Berkson (1951) and Hobson (1955) are the results expressed in terms of the international standard for HCG The curves obtained are of the same general shape as those reported by previous investigators who expressed results in 'rat and 'mouse units but are much more meaningful from the quantitative point of view

In Fig 16 are shown the mean urinary and serum levels of HCG throughout normal pregnancy Fig 17 presents observations of the urinary excretion made in a small number of individual subjects

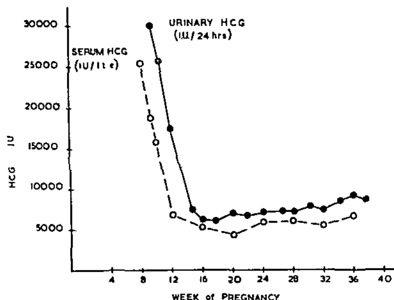



Fig 16

Mean estimations of urinary and serum HCG throughout normal pregnancy (*From Loraine* 1957)

Very large amounts of HCG appear in blood and urine during the first trimester of normal pregnancy This constitutes the so called peak period Readings obtained at this time are very variable from one subject to another and may vary from 20,000 to 100 000 i u per twenty four hours of urine or per litre of serum The time at which the HCG readings begin to decrease is also very variable, but usually the peak period has passed by the fifteenth week of pregnancy In the second and third trimesters the figures for both serum and

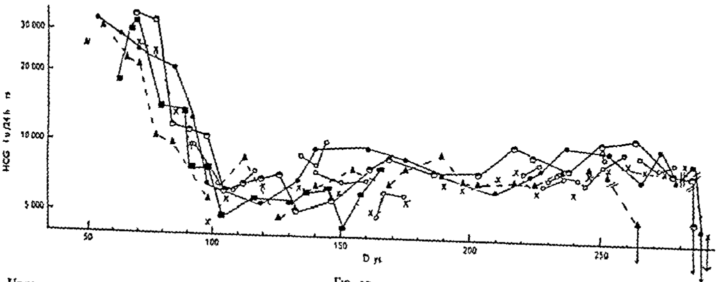



Fig 17

Urinary excretion of HCG throughout normal pregnancy in individual subjects Open circles denote scattered readings in various patients other signs denote individual cases transverse lines indicate date of delivery The duration of pregnancy is calculated from the first day of the last menstrual period (*From Loraine* 1950 *a*)

urine are much more constant than in early pregnancy. Loraine (1950 *a*, 1957) found that at this time the values lay in a range between 4 000 and 11 000 i.u. (P=0.99) and suggested that readings consistently outside these limits should be regarded as pathological. Fig. 16 illustrates the very close quantitative relationship between urine and serum levels of HCG. This subject will be more fully discussed in the section dealing with the renal clearance of the hormone (p. 91).

The exact time of appearance of HCG in the blood and urine of pregnant women is not yet known with certainty. Aschheim and Zondek (1927) originally reported that the substance could be detected in urine within two weeks after the first missed menstrual period. Levin (1941) studied the gonadotrophin excretion in a patient in whom pregnancy supervened in the course of a normal menstrual cycle. He found that the titre began to increase on the twenty-fourth day of the cycle and that by the thirtieth day the output had risen approximately a hundredfold; twelve days later the excretion was some 3 000 times higher than that usually encountered in normally menstruating women.

At the time of writing, HCG assays by reliable methods have been reported in only a few cases of multiple pregnancy. In a proportion of the cases studied the excretion has been significantly higher than in single pregnancy (Fosco, 1943; Jones *et al.*, 1944).

## 2 Hydatidiform Mole

It has been known for over twenty-five years that patients with hydatidiform mole tend to excrete abnormally large quantities of HCG. Earlier investigations suggested that such cases could be differentiated from normally pregnant women on the basis of HCG assays alone. For many years the criteria proposed by Zondek (1931) were generally accepted. These stated (*a*) that a concentration of HCG in a morning specimen of urine greater than 50 000 mouse units per litre was suggestive of either mole or chorionepithelioma, (*b*) that levels between 100 000 and 200 000 units made the diagnosis probable, and (*c*) that concentrations of 200,000 units or more were virtually diagnostic of one or other of these conditions. Later work, however, in which results of HCG assays were expressed in international units showed beyond all doubt

that the overlap of readings between normally pregnant women on the one hand and cases of mole and chorion epithelioma on the other was greater than was previously supposed and that for this reason Zondek's original classification could no longer be regarded as valid

One of the most detailed investigations so far reported is that of Hamburger (1944) who studied the HCG excretion in twenty patients with hydatidiform mole and expressed his results in international units He found that the output in individual subjects ranged from 6 000 to 20 000,000 i u per litre, but that in over 80 per cent of the cases the readings were above 300 000 i u per litre In a series of normally pregnant women studied at the same time the mean excretion was much lower than in the cases of hydatidiform mole However, the difference between the two groups was by no means clear cut as indicated by the fact that a small proportion of the normal subjects (8 per cent of the series) showed urinary HCG readings greater than 300,000 i u per litre From these observations Hamburger (1944) concluded that the quantitative determination of HCG in urine was of only limited value in the diagnosis of hydatidiform mole The information obtained by such assays may be suggestive but cannot be definitely diagnostic of the condition

In uncomplicated cases of mole removal of the latter causes a sharp decrease in the urinary excretion of HCG In Hamburger's series of cases assays were conducted one month three months and six months after evacuation of the mole In one month the titre had fallen considerably compared with the pre operative values and was generally well below 30,000 i u per litre in three months activity had disappeared from the urine in 91 per cent of cases and in six months this figure had risen to 100 per cent In a large series of cases recently studied by Hobson (1955) complete evacuation of the mole caused disappearance of gonadotrophic activity from the urine within one month

The pattern of HCG excretion is very different in cases of hydatidiform mole in whom complications such as retained molar fragments or chorionepithelioma supervene Under such circumstances serial HCG assays may be of considerable diagnostic and prognostic value According to Hamburger (1944) the presence of complications should be suspected if

the excretion of HCG one month post operatively exceeds 30 000 i u per litre If such a titre is found serial HCG determinations at weekly intervals should be performed, and if a steady rise in excretion occurs it is reasonable to assume that complications have developed If the output of HCG one month after removal of the mole is already higher than the pre operative value, the presence of a complication is almost certain and appropriate treatment should be instituted

### 3 Chorionepithelioma of the Uterus

This is a relatively rare uterine tumour Fifty per cent of cases follow hydatidiform mole 25 per cent follow abortion and 25 per cent occur after a full time pregnancy The tumour consists of actively proliferating chorionic tissue and is associated with abnormally high levels of HCG in blood and in urine From the quantitative point of view the HCG excretion in cases of chorionepithelioma is similar to that found in patients with hydatidiform mole and it is not possible to differentiate the two conditions by hormone assay alone The diagnostic value of HCG assays in cases of hydatidiform mole complicated by chorionepithelioma has been discussed above

### 4 Chorionepithelioma of the Testis

This is a rare highly malignant tumour which is rapidly invasive Metastases may occur from a relatively small primary growth and these may produce the first symptoms which call attention to the disease The tumour is derived from pluripotential undifferentiated epithelial cells

All investigators agree that in patients with this type of neoplasm large quantities of HCG appear in blood and urine The subject of hormone excretion in the various types of testicular tumour has been reviewed by Twombly (1944) and by Hamburger (1946) According to Twombly (1944) the presence of HCG in urine usually provides evidence that the tumour is radio resistant and that the prognosis for the patient is very poor Hamburger (1946) succeeded in demonstrating HCG activity in extracts of tumour tissue and concluded that the neoplasm itself and not the anterior pituitary was responsible for the elaboration of the hormone

Various workers have reported that patients with chorion epithelioma of the testis excrete quantities of HCG comparable

to those found in cases of hydatidiform mole (Ferguson 1933, Furuhjelm 1940) However at the time of writing little quantitative information is available in the literature regarding HCG excretion in this type of tumour The subject requires reinvestigation using more reliable assay methods

## 5 Fœtal Death

Quantitative assays of HCG may be of diagnostic and prognostic value in relation to the fate of the fœtus (Chosson and Donnet 1934 Rakoff, 1940) Fœtal death is indicated by a progressive fall in excretion values to levels not detectable by current assay methods In the investigation of a case of suspected fœtal death serial estimations should be undertaken HCG may continue to be secreted by the chorionic tissue for some time after the fœtus has died Accordingly a positive pregnancy test obtained at or near the time of suspected fœtal death is not of itself proof that the fœtus is still viable

## 6 Pre-eclamptic Toxæmia

Numerous workers have estimated HCG in the blood and urine of toxæmic women but in most investigations reported the results have been expressed in animal units and the assay methods employed have not been reliable From the clinical point of view interest has been focused on the possible value of serum and urinary HCG estimations as a prognostic index in such cases

Smith and Smith (1934, 1939 1941) using the corpora lutea assay method in rats were the first to note that a proportion of pre eclamptic patients (over 70 per cent of their series) showed abnormally large quantities of HCG in the serum when compared with normally pregnant women The rise in serum HCG was often observed some weeks before the development of pre eclampsia and the Smiths suggested that this finding might serve as a warning of impending toxæmia In later work however (Smith and Smith 1948) they were forced to the conclusion that the elevation of HCG bore little or no relationship to the severity of the disease and was therefore of little value in prognosis Taylor and Scadron (1939) employing the same method of assay as that used by the Smiths reported high concentrations of HCG in the urine and serum

of a proportion of their cases but in many toxæmic women the readings were entirely normal throughout the period of investigation Watts and Adair (1943) performed HCG assays in the urine of pre-eclamptic and eclamptic patients and of cases of essential hypertension in pregnancy It was shown that the mean excretion of HCG in pre-eclamptic toxæmia and eclampsia was significantly higher than in normal pregnancy but that cases of essential hypertension in pregnancy also excreted significantly higher amounts of HCG Watts and Adair concluded that abnormalities in HCG secretion *per se* did not play an important part in the ætiology of pre-eclamptic toxæmia

A reinvestigation of the problem was undertaken by Loraine and Matthew (1950) who used the prostatic weight method to assay HCG in blood and urine Observations were made in twenty nine patients with pre eclamptic toxæmia nine with essential hypertension in pregnancy and five with essential hypertension with superimposed toxæmia The pre eclamptic patients were further subdivided into three groups—severe moderate and mild The assessment of the severity of the toxæmia was based mainly on the rapidity of evolution of the disease and on its response to therapeutic measures Cases were said to be mild if the symptoms subsided under treatment and the patient proceeded uneventfully to term The disease was regarded as moderate when there was no appreciable change in the condition in spite of therapy In the severe group the course of the disease was fulminating in some eclamptic fits occurred and in all cases there was an urgent indication for the interruption of pregnancy For a diagnosis of essential hypertension in pregnancy it was necessary to have the history of hypertension prior to the onset of pregnancy or from the earliest weeks of pregnancy Another group presented features both of essential hypertension and pre-eclamptic toxæmia in these patients hypertension was already present in early pregnancy and the symptoms of pre eclamptic toxæmia appeared after the twentieth week

The results of HCG assays in the five groups of cases are shown in Figs 18 to 22 and in Table V

It was found that in severe pre-eclamptic toxæmia the mean urinary excretion and serum concentration of HCG were significantly higher than in normal pregnancy ($P < 0{\cdot}01$) but

30000
20000
HCG in i u
10 000
NORMAL RANGE
5,000
35 36 37 38 39 40
Weeks of pregnancy

Fig 18

HCG levels in severe pre eclamptic toxæmia Open circles denote urinary excretion closed circles denote serum concentration The estimations for each individual patient are grouped together (*From Loraine and Matthew* 1950)

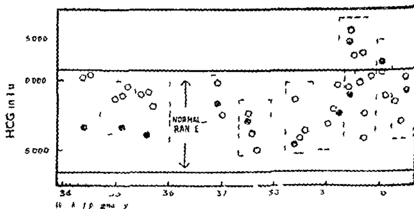



Fig 19

HCG levels in moderate pre eclamptic toxæmia Open circles denote urinary excretion closed circles denote serum concentration The estimations for each individual patient are grouped together (*From Loraine and Matthew* 1950)

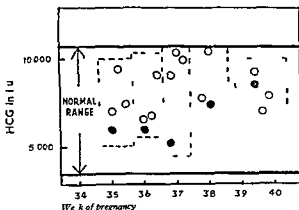



FIG 20

HCG levels in mild pre-eclamptic toxæmia Open circles denote urinary excretion closed circles denote serum concentration The estimations for each individual patient are grouped together (*From Loraine and Matthew* 1950)

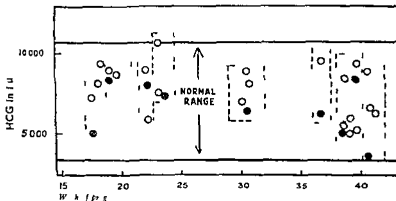



FIG 21

HCG levels in essential hypertension in pregnancy Open circles denote urinary excretion closed circles denote serum concentration The estimations for each individual patient are grouped together (*From Lor ine and Matthe* 1950)

Fig 18

HCG levels in severe pre-eclamptic toxæmia. Open circles denote urinary excretion, closed circles denote serum concentration. The estimations for each individual patient are grouped together. (*From Loraine and Matthew* 1950)

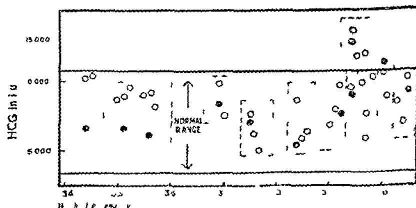



Fig 19

HCG level in moderate pre-eclamptic toxæmia. Open circles denote urinary excretion, closed circles denote serum concentration. The estimations for each individual patient are grouped together. (*From Loraine and Matthew* 1950)

TABLE V

URINARY EXCRETION AND SERUM CONCENTRATION OF HCG IN NORMAL, TOXÆMIC AND HYPERTENSIVE PATIENTS

*(From Loraine and Matthew 1950)*

| Type of Case | Number of Patients | Number of Patients with High HCG | Urinary HCG I U per Twenty four Hours | | | Serum HCG I U per Litre | | |
|---|---|---|---|---|---|---|---|---|
| | | | Number of Estimations | Mean | Standard Deviation | Number of Estimations | Mean | Standard Deviation |
| Severe pre-eclamptic toxæmia | 12 | 8/12 | 30 | 13 ,10 | 5 420 | 12 | 13 050 | 7 160 |
| Moderate pre eclamptic toxæmia | 12 | 2/12 | 31 | 8 840 | 2 370 | 12 | 8 030 | 2 860 |
| Mild pre-eclamptic toxæmia | 5 | ,/5 | 14 | 8 260 | 1 610 | , | 6 4,0 | 1 00 |
| Essential hypertension (uncomplicated) | 9 | 0/9 | 22 | 7 490 | 1 620 | 9 | 6 250 | 1 400 |
| Essential hypertension with uper imposed toxæmia | 5 | 0 5 | 14 | 8 ,60 | 1 820 | 5 | 7 950 | 1 110 |
| Normal control subjects | 12 | 0/12 | 15 | 8 3 0 | 1 490 | 15 | 6 400 | 1 460 |

in none of the other groups of patients was a significant difference observed No correlation could be demonstrated between the high readings of HCG on the one hand and any clinical feature

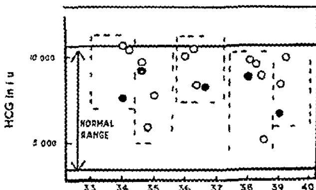



Fig 22

HCG levels in essential hypertension with superimposed toxæmia Open circles denote urinary excretion closed circles denote serum concentration The estimations for each individual patient are grouped together (*From Loraine and Matthew* 1950)

such as the presence of œdema, the degree of albuminuria and the height of the blood pressure on the other It was concluded that at the present time routine assay of the urinary and serum HCG would not be of much assistance to the clinician in the management of cases of pre eclamptic toxæmia

### 7 Hyperemesis Gravidarum

Various investigators including Anselmino and Hoffman (1936), Rakoff (1940) and Schoeneck (1942), have claimed that patients with hyperemesis gravidarum tend to show abnormally large quantities of HCG in blood and urine The most detailed study is probably that of Schoeneck (1942) who attempted to adapt the Friedman test for the quantitative assay of HCG in urine He reported that patients with hyperemesis excreted significantly larger quantities of HCG than did normally pregnant women and that a positive correlation existed between the severity of the vomiting and the HCG output These results were not confirmed by Loraine (1949 *a*) who found that the HCG excretion in such patients lay within the normal range for the duration of pregnancy

incidence of premature delivery and pre eclamptic toxæmia was nil

(*d*) Stilbœstrol and progesterone corrected the so called 'hormonal imbalance and restored the HCG levels to normal When hormonal therapy was employed the fœtal survival rate rose sharply and the incidence of premature delivery and pre eclamptic toxæmia fell correspondingly

In a series of ninety eight patients showing hormonal imbalance' and not receiving therapy the fœtal survival rate was only 52 per cent, the incidence of premature delivery was 33 per cent and of pre eclamptic toxæmia 33 per cent These figures were in marked contrast to the results obtained in 380 patients in whom the 'hormonal balance was corrected by substitution therapy In this latter group the fœtal survival rate rose to 90 per cent, premature delivery occurred in 17 per cent and the incidence of pre eclamptic toxæmia fell to 7 per cent

Other groups of investigators have failed to confirm White s claims with regard to the diagnostic and prognostic value of HCG assays in pregnant diabetics For example Keltz *et al* (1950) in a series of thirty patients, were unable to show that the serum HCG concentration bore any relationship either to the occurrence of fœtal death or to the development of pre eclamptic toxæmia In the experience of these workers com bined therapy with œstrogens and progesterone did not affect the serum concentration of HCG in the third trimester of diabetic pregnancy

Loraine and Matthew (1954) have recently studied the urinary excretion and serum concentration of HCG in fifty two pregnant diabetics The hormone was assayed by the prostatic weight method in rats and results were expressed in terms of the international standard Readings for both serum and urine were considered to be abnormally high if they were consistently above 11 000 I U in the second and third trimesters of pregnancy In the earlier stage of the investigation oral stilbœstrol was administered to those patients showing an abnormally high excretion of urinary HCG During this time six out of fourteen cases were so treated In the later stages of the study no hormonal therapy was given irrespective of the HCG readings in blood and urine

The results obtained by Loraine and Matthew (1954) did

## 8 Diabetic Pregnancy

Although the introduction of insulin greatly reduced the maternal mortality rate in diabetes mellitus, the fœtal mortality has not shown a corresponding decline The high fœtal loss rate remains the main problem in pregnant diabetics and in some series of cases the mortality has reached 50 per cent It is not surprising, therefore, that considerable interest has been aroused by the claims made by White and her collaborators in Boston (White, 1947, 1952, White and Hunt, 1943) that a very significant reduction in the fœtal mortality rate in diabetic pregnancy (from 50 per cent to approximately 10 per cent) can be obtained with hormonal therapy The therapy for White's patients consisted of the parenteral administration of œstrogens and progesterone, these substances were given to correct a so called hormonal imbalance which was characterised by an abnormally high serum concentration of HCG and an abnormally low excretion of urinary pregnanediol HCG was assayed by the corpora lutea method in rats The method of assay of pregnanediol is described in Chapter X along with the results obtained

White (1952) has published her experience with this therapy in 525 pregnant diabetics studied over a fifteen year period Stilbœstrol and progesterone (Proluton) were administered parenterally in a gradually increasing dosage throughout pregnancy to those patients showing hormonal imbalance The dosage schedule employed was similar to that used by the Smiths in the treatment of patients with pre eclamptic toxæmia Approximately 10,000 estimations of serum HCG were made in the 525 cases From her studies White (1952) drew the following conclusions

(*a*) Ninety one per cent of the patients showed an abnormally high serum HCG concentration

(*b*) Estimation of the serum HCG was of considerable diagnostic and prognostic value in these patients because a rise of serum HCG often preceded obstetrical complications by some weeks

(*c*) Only in patients with an abnormally high serum HCG concentration did obstetrical accidents and complications occur In forty seven patients in whom the serum level was within normal limits, the fœtal survival rate was 96 per cent and the

Fig 23

Urinary excretion of HCG in a pregnant diabetic treated with stilbœstrol The small figures indicate dosages (milligrams per day) and are placed at the date the treatment was started (*From Loraine* 1953)



Fig 4

Urinary excretion of HCG in a pregnant diabetic treated with stilbœstrol The small figures indicate dosages (milligrams per day) and are placed at the date the treatment was started (*From Loraine* 1953)

not agree in three respects with those of White and her collaborators

(*a*) It was found that, as compared with White's series, a much smaller proportion of diabetic patients showed abnormally high HCG readings in blood and urine Of the forty nine patients in whom estimations of urinary HCG were undertaken, only fourteen (29 per cent ) showed abnormally high figures while of the thirty seven patients in whom the serum concentration of the hormone was estimated only ten (27 per cent) were above the normal range In the total series of fifty two patients high levels of HCG either in urine or in serum were found in seventeen cases only (33 per cent )

(*b*) When the series of cases studied in Edinburgh was analysed to show the relationship of HCG levels to obstetric complications, it was found that fœtal death pre eclamptic toxæmia and premature delivery were just as likely to occur in pregnant diabetics with normal HCG readings as in those with abnormal levels

(*c*) Stilbœstrol produced only a temporary fall in the HCG excretion in pregnant diabetics (Loraine 1949 *b*) Consistent depression of the output by prolonged œstrogen therapy was *not obtained even when large doses of stilbœstrol (100 to 200 mg* per day) were administered The initial depression was soon followed by an escape phenomenon and the readings tended to return to their original level while the patient was still receiving œstrogen therapy Typical results in two pregnant diabetics treated with stilbœstrol are shown in Figs 23 and 24

In Fig 25 is shown the urinary excretion of HCG in twenty five pregnant diabetics to whom hormonal treatment was not given It will be noted that in this series of patients the majority of readings lay within the normal range

It can only be assumed that the differing results obtained by the various groups of investigators for the HCG levels in pregnant diabetics must depend to a large extent on the methods of assay employed Mention has already been made of the grave disadvantages of the rat corpora lutea test as used by White and her collaborators In the assays conducted by these workers the relative potency was not expressed in terms of the international standard for HCG but in ' rat units per 100 ml of serum Accordingly it is doubtful if any quantitative significance can be attached to the results obtained

## RENAL CLEARANCE OF HCG IN NORMAL AND ABNORMAL PREGNANCY

As noted previously (p 74) the urinary excretion and serum concentration of HCG fluctuate characteristically during normal pregnancy This fluctuation might represent alterations in the rate of formation, in the rate of destruction or in the rate of excretion of the hormone In an attempt to gain further information on this problem Gastineau *et al* (1949) studied the renal clearance of HCG in normally pregnant women and in patients with hydatidiform mole and testicular tumours The method of assay of HCG depended on the ovarian hyperæmia response in rats, renal clearance was calculated by the formula UV/B[1] and was expressed as millilitres per minute These workers reported a mean clearance in normal pregnancy of 0 36 ml per min and also found that the mean clearance in the first trimester of normal pregnancy did not differ significantly from that obtained in the second and third trimesters From available data Gastineau *et al* (1949) calculated that the renal clearance in a series of patients studied by Smith and Smith (1939) was 0 85 ml per min and that obtained by Taylor and Scadron (1939) was 0 44 ml per min Such very low figures for clearance are typical of proteins in general

Loraine (1950 *b*) using the prostatic weight method of assay, estimated the HCG clearance in normally pregnant women patients with pre eclamptic toxæmia, pregnant diabetics, cases of essential hypertension in pregnancy and patients with hypertension and superimposed toxæmia In all groups of cases the mean clearance was found to be less than 1 ml per min In normal and diabetic pregnancy consistent figures for clearance were obtained in the three trimesters Accordingly it appeared reasonable to conclude that variations in HCG levels during pregnancy probably represented alterations in the rate of production and destruction of the hormone and were not the result of differences in the renal excretion

The pre eclamptic patients were classified on a clinical

[1] U = Concentration of the hormone in the urine
V = Volume of urine secreted per minute
B = Concentration of the hormone in the serum

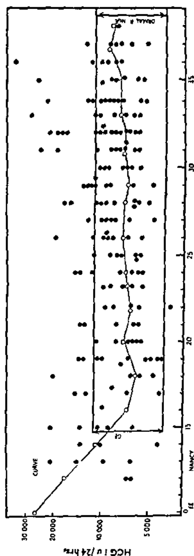



FIG 25

Urinary excretion of HCG in twenty five pregnant diabetics without hormonal therapy—total number of estimations = 230 (*From Loraine* 1953)

## RENAL CLEARANCE OF HCG IN NORMAL AND ABNORMAL PREGNANCY

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The pre eclamptic patients were classified on a clinical

[1] U = Concentration of the hormone in the urine
V = Volume of urine secreted per minute
B = Concentration of the hormone in the serum

basis into three categories—mild, moderate and severe In the mild and moderate cases the mean clearance did not differ significantly from that in normal pregnancy Similar results were obtained in patients with uncomplicated essential hypertension and in cases showing essential hypertension with superimposed toxæmia In the severe pre eclamptic group, however the mean clearance was significantly lower than that in normal pregnancy This observation is probably merely another indication of the renal damage frequently found in severe cases of pre eclamptic toxæmia

In the studies reported by Loraine (1950 *b*) a close quantitative similarity was noted between the urinary and serum concentration of HCG In all types of case the mean urine/serum concentration ratio was approximately unity In the series of patients investigated by Gastineau *et al* (1949) the figures obtained for the urinary excretion of HCG were quantitatively very similar to those obtained by Loraine (1950 *b*) but their serum concentrations were much higher, averaging 3 06 times the concentration in the urine Diczfalusy and Loraine (1955) suggested that this discrepancy might result from the use of different assay methods by the two groups of workers The precision of the ovarian hyperæmia test as used by Gastineau *et al* (1949) is relatively low (Table III) and it is difficult to exclude the possibility that the figures obtained by this method for the concentration of HCG in pregnancy serum may be erroneously high Such a criticism does not appear to apply to results obtained using the prostatic weight technique

## CONCENTRATION OF HCG IN THE PLACENTA IN NORMAL AND ABNORMAL PREGNANCY

Smith and Smith (1935) found that placentæ from toxæmic patients had a significantly greater concentration of HCG than those from normally pregnant women Various investigators have demonstrated a considerably higher HCG concentration in placentæ obtained during the early weeks of gestation when compared with those examined in late pregnancy By far the most careful study of the HCG concentration in normal human placentæ is that of Diczfalusy (1953) This worker who expressed results in international units found that the placental

concentration of HCG was maximal in the second and third months of pregnancy (corresponding to the peak period in blood and urine), fell rapidly in the third and fourth months and from the fifth month onwards was relatively constant remaining below 20 i u per g

Loraine and Matthew (1953) estimated the placental concentration of HCG in normal diabetic and toxæmic pregnancy They found that the placental HCG concentration did not

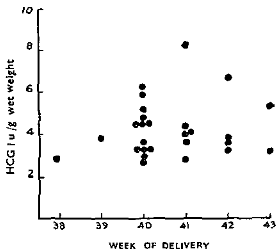



Fig 26
Placental concentration of HCG in subjects with an uncomplicated pregnancy and vaginal delivery (*From Loraine and Matthew 1953*)

differ significantly between normal patients delivered by the vaginal route and patients delivered by Cæsarean section for purely obstetrical indications In cases with moderate and severe pre eclamptic toxæmia and in diabetic pregnancy, however the mean figure for the HCG concentration was significantly higher than that in the control series but in patients with mild pre eclamptic toxæmia the mean figure did not differ significantly from that in the control series The results obtained in the different types of case are shown in Figs 26 to 29 and in Table VI

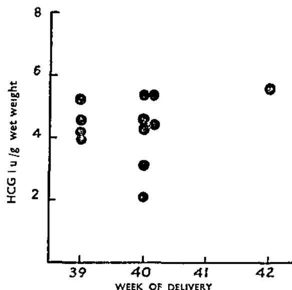



Fig 27

Placental concentration of HCG in patients delivered by Cæsarean section for obstetric complications (*From Loraine and Matthew* 1953)

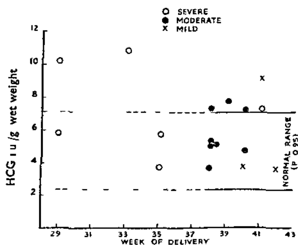



Fig 28

Placental concentration of HCG in patients with pre eclamptic toxæmia (*From Loraine and Matthew* 1953)

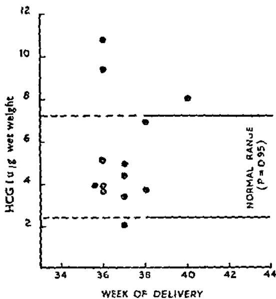



Fig 29
Placental concentration of HCG in pregnant diabetics
*(From Loraine and Matthew 1953)*

## Table VI

### Placental Concentration of HCG in Normal and Abnormal Pregnancy

*(From Loraine and Matthew 1953)*

| Type of Case | Number of Patients | Mean HCG Concentration I U per g Wet Weight | Standard Deviation of the Logarithm | Range (P = 0 95) I U per g Wet Weight |
|---|---|---|---|---|
| Uncomplicated pregnancy with vaginal delivery | 27 | 4 11 | 0 1 | 2 39 to 7 06 |
| Caesarean section (obstetrical abnormalities) | 12 | 4 8 | 0 13 | 2 39 to 7 73 |
| Severe pre eclamptic toxemia and eclampsia | 6 | 6 89 | 0 16 | 3 34 to 14 19 |
| Moderate pre eclamptic toxaemia | 8 | 70 | 0 11 | 3 41 to 9 33 |
| Mild pre eclamptic toxaemia | 3 | 5 11 | 0 3 | 3 03 to 14 33 |
| Diabetes mellitus | 14 | 5 16 | 0 2 | 1 9 to 13 93 |

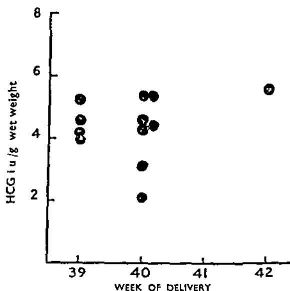



FIG 27

Placental concentration of HCG in patients delivered by Cæsarean section for obstetric complications (*From Loraine and Matthew* 1953)

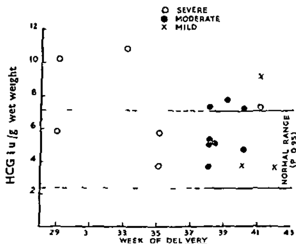



FIG 28

Placental concentration of HCG in patients with pre eclamptic toxæmia (*From Loraine and Matthew* 1953)

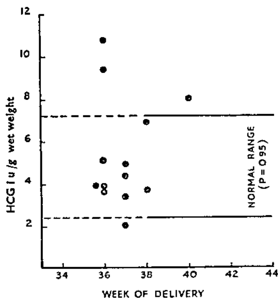



FIG 29
Placental concentration of HCG in pregnant diabetics
(*From Loraine and Matthew* 1953)

## TABLE VI

## PLACENTAL CONCENTRATION OF HCG IN NORMAL AND ABNORMAL PREGNANCY

(*From Loraine a d Matthew* 1953)

| Type of Case | Number of Patients | Mean HCG Concent a tion I U per g Wet W ght | Standard Dev ation of the Logarithm | Range (P=0 95) I U per g Wet Weight |
|---|---|---|---|---|
| Uncomplicated pregnancy with vaginal del very | 27 | 4 11 | 0 1 | 39 to 7 06 |
| Cæsarean sect on (obstetr cal abnormal ties) | 1 | 4 8 | 0 13 | 39 to 7 73 |
| Severe pre eclamptic tox æmia and eclamp a | 6 | 6 89 | 0 16 | 3 34 to 14 19 |
| Moderate pre eclampt c toxæmia | 0 | 70 | 0 11 | 3 44 t 9 33 |
| Mild pr eclampti toxæm a | 3 | 5 11 | 0 3 | 3 03 to 14 39 |
| D abetes mell tus | 14 | 5 16 | 0 2 | 1 9 to 13 93 |

## Summary and Conclusions

Many bio assay methods for the quantitative determination of HCG in body fluids have been described The most satisfactory techniques at present available are those depending on the prostatic weight in rats or on the expulsion of spermatozoa in amphibia The success of the ovarian hyperæmia test in rats appears to depend to a large extent on the strain of animal employed Care is necessary in the selection of assay methods for estimating HCG in pregnancy serum in order to avoid an overestimate of potency

In hydatidiform mole and chorionepithelioma of the uterus HCG levels in blood and urine are generally higher than those in normally pregnant women, but the difference between the two groups is not clear cut

In severe pre eclamptic toxæmia the mean figures for the urinary excretion and serum concentration of HCG are significantly higher than those in normal pregnancy, but in mild and moderate pre eclamptic toxæmia and in essential hypertension in pregnancy the figures do not differ significantly from those in normal pregnancy There is no apparent correlation between the HCG levels and any clinical feature of the disease

In diabetic pregnancy approximately 30 per cent of patients show abnormally high readings of HCG in serum and urine There is however, no correlation between the HCG levels on the one hand and the medical and obstetrical findings on the other Stilbœstrol when administered to diabetic women causes a transient fall in the urinary excretion of HCG

In all cases the mean renal clearance of HCG is less than 1 ml per min In normal and diabetic pregnancy constant figures are obtained in the three trimesters of pregnancy The mean clearance in severe pre eclamptic toxæmia is significantly lower than in normal pregnancy

In moderate and severe pre eclamptic toxæmia and in diabetic pregnancy the mean placental concentration of HCG is significantly higher than in normal subjects

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## CHAPTER IV

# *Thyrotrophin*

## INTRODUCTION

THIS hormone is elaborated by the anterior lobe of the pituitary and controls the activity of the thyroid gland Other names for thyrotrophin are the *thyrotrophic hormone* and the *thyroid stimulating hormone* (TSH)

### 1 Chemical Nature of Thyrotrophin

Thyrotrophin was first extracted from bovine pituitary glands by Loeb and Bassett in 1930 Since then considerable progress has been made in the purification of the hormone The preparation made by Ciereszko and White (1942) was a white powder which was freely soluble in water the substance was shown to behave as a pure protein in electrophoretic and solubility studies The molecular weight of the hormone was estimated to be 10 000

### 2 Thyrotrophin and Exophthalmos producing Substance (EPS)

It has been known for many years that anterior pituitary extracts when administered to animals produce both thyroid hyperplasia and exophthalmos and it has been generally assumed that the two effects are due to one and the same substance namely, thyrotrophin This view has recently been challenged by Dobyns and his associates (Dobyns 1946 , Dobyns and Steelman 1953) as a result of their experiments on pituitary extracts These workers assayed TSH by a variety of methods in day old chicks and EPS by the degree of exophthalmos produced in the Atlantic minnow *Fundulus heteroclitus* They found that in many cases the two activities did not run parallel and showed that it was possible to separate TSH from EPS in these extracts by a method depending on the differential solubility of the two substances in trichloracetic acid

In view of these observations it is obvious that an attempt

should be made to develop a technique for the estimation of exophthalmos producing substance in the body fluids of man Assays of EPS and TSH should if possible be conducted on the same patients The information obtained from such assays might well provide valuable information in relation to the aetiology of clinical exophthalmos

### 3 The International Standard for Thyrotrophin

This was recently established by the World Health Organisation (Mussett and Perry, 1955) One international unit is defined as the activity contained in 13 5 mg of the international standard Results of all assays for thyrotrophin should now be expressed in terms of the standard preparation Animal units such as those described by Junkmann and Schoeller (1932) and Heyl and Laqueur (1935) should no longer be used

## METHODS OF ASSAY OF THYROTROPHIN

A very large number of bio assay methods for TSH have been described in the last two decades Many of the techniques are capable of estimating the thyrotrophin content of pituitary extracts but few are sufficiently sensitive to measure the small quantities of the hormone present in human blood and urine There is at present a great need for a reliable method of TSH assay which would be suitable for relatively wide clinical application Such a procedure would enable clinicians to correlate the TSH levels in blood and urine with other objective assessments of thyroid function such as those obtained by radio isotope studies and by the estimation of the blood concentration of protein bound iodine These combined studies might well give new and interesting information regarding the aetiology of such conditions as primary Graves disease, secondary toxic nodular goitre, pre tibial myxoedema and various forms of hypothyroidism Furthermore such investigations might provide data by means of which new and more satisfactory forms of therapy could be introduced for patients suffering from these diseases

In the assay methods proposed by various investigators five main experimental animals have been used These are (1) the chick, (2) the guinea pig, (3) the rat (4) the mouse and (5) the

tadpole For further information on the present position of TSH assay the reader is referred to review articles by Turner (1950) Gaddum and Loraine (1950), Tala (1952), Lamberg (1953) and Wahlberg (1955)

## 1 Methods employing Chicks

The thyroid of the one day old or two day old chick is one of the most widely used test objects for the assay of thyrotrophin In general, assays in chicks are rather more sensitive than those in rats mice and guinea pigs However as emphasised by Lamberg (1953) and by others such methods require rigid standardisation of technique during their performance Among the many factors which will influence the response are the strain weight sex and age of the chick, the environmental temperature the intensity of illumination the geographical location and the diet The following indices of response in the chick thyroid will be considered

(*a*) THYROID WEIGHT —TSH will increase the weight of the chick thyroid and this assay method was originally proposed by Smelser (1937) for the estimation of the thyrotrophic potency of beef pituitary extracts The method is time consuming and is too insensitive for use in the clinical field

(*b*) ACINAR CELL HEIGHT —In 1940 Rawson and Salter described a microhistometric method in which the end point of the assay depended on the ability of TSH to cause an increase in acinar cell height The technique is very laborious as over 100 successive acini must be examined in each section It has however, been claimed that the method is sufficiently sensitive to detect the presence of the hormone in human urine

(*c*) IODINE CONTENT —Thyrotrophin increases the uptake and turnover of iodine by the thyroid gland and various assay methods have been devised depending on this effect Most workers now employ radio active iodine ($^{131}I$) in preference to inert iodine and it is usually advisable to pre treat the chicks with thyroxine in order to inhibit the secretion of endogenous TSH One of the most promising methods is that described by Gilliland and Fraser (1953) This depends on the discharge of $^{131}I$ from the thyroid gland of the one day old male chick which has been pre treated with thyroxine The technique is both simple and rapid and is claimed to be capable of estimating TSH in the blood of normal subjects

(*d*) PHOSPHORUS UPTAKE —It is well known that TSH will increase the uptake of phosphorus by the thyroid gland Assay methods for the hormone based on the uptake of $^{32}$P have been described by Lamberg (1953) and by Crooke and Matthews (1953) Such techniques are rapid simple and reasonably specific, but it is doubtful if they are sufficiently sensitive for use in the clinical field

## 2 Methods employing Guinea pigs

Observations can be based on the weight of the thyroid on histological changes on the uptake of $^{32}$P and on the discharge of $^{131}$I The following methods will be considered

(*a*) ACINAR CELL HEIGHT —This is a microhistometric procedure which was introduced by Rawson and Starr (1938) and depends on the ability of TSH to cause an increase in the height of the epithelial cells lining the acini In its original form the technique is too insensitive for studies in man

(*b*) PERCENTAGE OF EPITHELIUM —Uotila and Kannas (1952) and Tala (1952) have recently described a method for the quantitative estimation of TSH which involves determination of the relative proportion of epithelium colloid and stroma in the thyroid These components are measured along crossed straight lines drawn at random in a section projected at high magnification The percentage of epithelium is believed to give a more satisfactory index of thyroid activity than the other components According to Tala (1952) this method is extremely sensitive and will detect as little as 0 00001 of a Junkmann Schoeller unit of TSH[1] Clinical studies with this technique will be awaited with interest

(*c*) INTRACELLULAR COLLOID DROPLETS —de Robertis and del Conte (1944) developed a very sensitive assay method for TSH depending on the determination of the number of colloid droplets in the cells of the guinea pig thyroid This technique is almost certainly not specific for TSH and should no longer be used

(*d*) RADIO ACTIVE IODINE DISCHARGE —In this procedure which has been devised by Adams and Purves (1955) the

[1] A Junkmann Schoeller Unit (J S U) is defined as the amount of thyrotrophin required to produce after three daily injections a definite histological reaction in one out of two guinea pigs weighing between 100 and 150 g With the introduction of the international standard for thyrotrophin such a unit should no longer be used

secretion of TSH from the animal s own pituitary is inhibited by the administration of thyroxine and the thyroidal iodine is labelled using $^{131}$I In animals so treated the rise in the radio activity of the blood following the intravenous injection of thyrotrophin is a measure of the amount of thyrotrophin injected The method is simple, specific and reasonably precise By its use the authors have been able to detect TSH activity in the plasma of thyroidectomised rats and in that of rats treated with methyl thiouracil It is not yet known whether the technique is suitable for studies in patients

### 3 Methods employing Rats

In general, assays employing intact or hypophysectomised rats are less sensitive than those depending on chicks guinea pigs and tadpoles, and for this reason such techniques have not found wide use in clinical studies Various indices of response have been used including thyroid weight thyroid histology $^{131}$I uptake, $^{3}$ P uptake and the increase in basal metabolic rate

### 4 Methods employing Mice

Querido *et al* (1953) have described an assay method for TSH which depends on the $^{131}$I uptake of the thyroid in female mice which have been pre treated with iodocasein in order to reduce glandular activity The method is simple and precise but is relatively insensitive It is however possible to employ it in clinical studies provided that blood concentrates rather than untreated serum are administered to the test animals

### 5 Methods employing Tadpoles

D Angelo and his co workers (D Angelo *et al*, 1942 1951) have used the starved or stasis *Rana pipiens* tadpole for the assay of thyrotrophin When tadpoles are starved thyroid atrophy and metamorphic stasis ensue resumption of development occurs after the intraperitoneal administration of either thyroid hormone or TSH In the assay method of D Angelo *et al* (1942) two end points are measured, viz, thyroid cell height and hind limb extrusion The former is considered to be produced by TSH alone and the latter by

a combination of TSH and thyroid hormone A typical dose effect curve using both indices of response is shown in Fig 30

It is claimed that in a given sample of blood the concentrations of both hormones can be determined and that this method

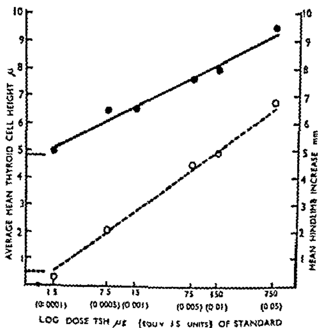



Fig 30

Log dose response curves indicating the increase in thyroid cell height (continuous line) and the extension of hind limbs (interrupted line) in the stasis tadpole with increasing doses of TSH Cell height at each point is based on counts in 6 to 9 animals horizontal bars on ordinate represent values in control animals (*From D Angelo et al* 1951)

may be used to accumulate information on the interrelationships between the pituitary and the thyroid in health and disease

This technique is time consuming and laborious the experimental period being fifteen days However, it has the advantage of great sensitivity and will measure as little as 0 0005 of a Junkmann Schoeller unit of TSH D Angelo *et al* (1951) and Asboe Hansen *et al* (1952) have used the stasis tadpole method to estimate the serum concentration of TSH in patients with normal and abnormal thyroid function. Their results will be discussed later in this chapter

### 6 General Conclusions regarding Thyrotrophin Assay Methods

None of the existing methods is suitable for routine clinical use, but a number of them may be of limited value in research centres The introduction of an international standard pre paration for TSH should make the assays more reliable The most promising tests from the point of view of their clinical application are those depending on $^{131}$I discharge in chicks and guinea pigs on the percentage of epithelium in the guinea pig thyroid and on the thyroid cell height in starved tadpoles The method depending on $^{131}$I uptake in mice can also be used in the clinical field if the blood extracts are concentrated prior to bio assay

## METHODS OF EXTRACTION OF THYROTROPHIN FROM BODY FLUIDS

Thyrotrophin is present in the blood and urine of human subjects in very low concentrations and accordingly bio assay methods of great sensitivity must be used in clinical studies Although it is claimed that certain methods are sufficiently sensitive to detect the presence of the hormone in untreated serum it is probable that more satisfactory results would be obtained if the blood and urine were extracted and con centrated prior to administration to the test animals At present little quantitative information is available on the yields of thyrotrophin obtained by the different extraction methods Further work is necessary on this important problem

### 1 Urine

Reports by earlier investigators that TSH activity could be detected following the administration of untreated urine to experimental animals were not subsequently confirmed and most workers now agree that concentration methods should be employed Many of the techniques proposed are the same as those used for the preparation of pituitary gonadotrophins from urine and include such methods as alcohol and acetone precipitation tannic acid precipitation and benzoic acid adsorption Jones (1939) in a careful study compared the efficacy of various extraction methods TSH was added to pooled urine and the urine was then extracted

a combination of TSH and thyroid hormone A typical dose effect curve using both indices of response is shown in Fig 30

It is claimed that in a given sample of blood the concentrations of both hormones can be determined and that this method

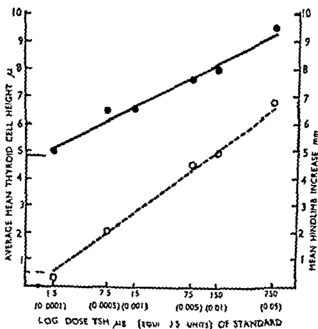



Fig 30

Log dose response curves indicating the increase in thyroid cell height (continuous line) and the extension of hind limbs (interrupted line) in the stasis tadpole with increasing doses of TSH Cell height at each point is based on counts in 6 to 9 animals horizontal bars on ordinate represent values in control animals (*From D Angelo et al* 1951)

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*the few investigations so far reported workers have generally* been unable to estimate thyrotrophin in the urine of normal individuals or in cases of thyrotoxicosis but have detected its presence in the urine of patients with myxœdema It is not yet known with certainty what proportion of thyrotrophin can be recovered from the urine after the parenteral administration *of the hormone to normal subjects or to patients with various* forms of thyroid disease

## 2 Studies on Blood

The most careful investigation of the serum concentration of TSH in normal and pathological conditions so far reported

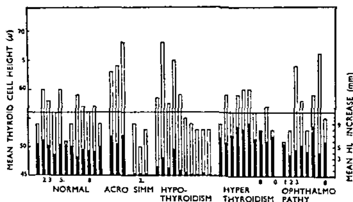



*Fig* 31

Comparison of metamorphic and thyroid stimulating activity of sera under normal and pathological conditions in man On the abscissæ are shown the individual cases comprising the normal series and the clinical groups of patients The height of the open bar indicates the mean thyroid cell height in the test animal The height of the darkened bar refers to the degree of hind limb extension produced by the same sample of serum The proportion of total increase due to the effect of the thyroid alone can be calculated by subtracting the hind limb extension corresponding to the thyroid cell height (*From D Angelo et al* 1951 )

is that of D Angelo *et al* (1951) These workers used the stasis tadpole technique by which it is possible to measure both TSH and thyroid hormone in a given sample of serum Their results are shown in Fig 31

It will be noted that in *normal* serum TSH activity although present was relatively low while thyroid hormone activity was relatively high In three cases of *acromegaly* blood titres

in different ways The potency of the final extracts was determined biologically using the thyroid of the one day old chick It was found that methods depending on simple alcohol or acetone precipitation gave more satisfactory yields of the hormone than those involving tannic acid precipitation or benzoic acid adsorption It would be of considerable interest to repeat these observations using the more sensitive bio-assay methods for TSH which have recently become available

**2 Blood**

D Angelo *et al* (1951) and Asboe Hansen *et al* (1952) have used the stasis tadpole technique to assay TSH and have found that this method is sufficiently sensitive to detect the presence of the hormone in untreated serum The total dose of serum administered per animal is approximately 0 2 ml spread over the six day injection period

Querido and his co workers (Querido *et al* 1953, 1955, Lameijer *et al* 1955) have described a method of assay for TSH depending on the accumulation of $^{131}I$ by the thyroid in mice which have been pre treated with iodocasein The technique is much less sensitive than that involving tadpoles and negative results are obtained when untreated serum is injected However these workers have developed methods for concentrating the serum and have been able to detect TSH activity in human blood by the administration of such concentrates to the test animals The method of extraction used was similar to that employed by Cohn *et al* (1946) in fractionation studies on plasma proteins and consisted of alcohol precipitation at low temperatures combined with *p*H adjustment By means of this technique it was possible to show that thyrotrophic activity when present was *entirely* confined to the $\beta$ globulin fraction

## THE CLINICAL APPLICATIONS OF THYROTROPHIN ASSAY

**1 Studies on Urine**

Little is at present known regarding the urinary excretion of TSH in health and disease and further information on this important subject must await the development of more satis factory methods of extraction and assay of the hormone In

the few investigations so far reported workers have generally been unable to estimate thyrotrophin in the urine of normal individuals or in cases of thyrotoxicosis, but have detected its presence in the urine of patients with myxœdema It is not yet known with certainty what proportion of thyrotrophin can be recovered from the urine after the parenteral administration of the hormone to normal subjects or to patients with various forms of thyroid disease

## 2 Studies on Blood

The most careful investigation of the serum concentration of TSH in normal and pathological conditions so far reported

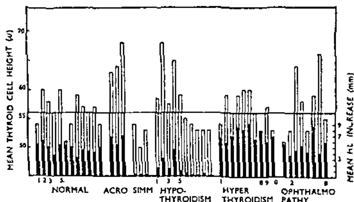



FIG 31

Comparison of metamorphic and thyroid stimulating activity of sera under normal and pathological conditions in man On the abscissæ are shown the individual cases comprising the normal series and the clinical groups of patients The height of the open bar indicates the mean thyroid cell height in the test animal The height of the darkened bar refers to the degree of hind limb extension produced by the same sample of serum The proportion of total increase due to the effect of the thyroid alone can be calculated by subtracting the hind limb extension corresponding to the thyroid cell height (*From D Angelo et al* 1951 )

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It will be noted that in *normal* serum TSH activity although present was relatively low while thyroid hormone activity was relatively high In three cases of *acromegaly* blood titres

of TSH were increased approximately eightfold over those in normal subjects while thyroid hormone levels were within the normal range Neither thyroid hormone nor TSH was demonstrable in the serum of three patients with *panhypopituitarism* In ten cases of *myxœdema* blood thyroid concentrations were consistently low, but TSH titres fluctuated greatly, being within, above or below the normal range In some of these cases hormone assays were conducted before and after treatment with thyroid extract, it was shown that clinical improvement of the condition was sometimes but not always associated with a return of the hormone levels to normal In ten cases of *thyrotoxicosis* abnormally high thyroid hormone concentrations were usually found in conjunction with normal TSH values In eight patients with *exophthalmic ophthalmoplegia* the blood levels of both thyroid hormone and TSH were essentially normal

Asboe Hansen *et al* (1952), using a similar assay method, have studied the serum concentration of TSH in normal subjects and in patients with exophthalmic ophthalmoplegia They were unable to demonstrate TSH activity in normal serum but found appreciable quantities of the hormone in nine out of ten cases of exophthalmic ophthalmoplegia The reason for the divergent findings reported by D Angelo *et al* (1951) on the one hand and by Asboe Hansen *et al* (1952) on the other is at present not clear but it is possible that the discrepancy will be explained on a methodological basis

## Summary and Conclusions

A large number of bio assay methods for thyrotrophin have been described but none of these is suitable for routine use in the clinical field Among the more promising techniques from the point of view of sensitivity are those depending on $^{131}I$ discharge in chicks and guinea pigs, on $^{131}I$ uptake in mice on the percentage of epithelium in guinea pigs and on the thyroid cell height in starved tadpoles

Further work is necessary on methods of extraction of TSH from blood and from urine

Abnormally high serum TSH levels have been reported in myxœdema and in acromegaly In Graves disease the values have usually been within the normal range

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CHAPTER V

# *Adrenocorticotrophin*

## INTRODUCTION

THIS hormone is elaborated by the anterior lobe of the pituitary and controls the secretory activity of the adrenal cortex. Other names for adrenocorticotrophin are *corticotrophin* and the *adrenocorticotrophic hormone* (ACTH).

### 1 Chemical Nature of ACTH

There has been much controversy on this subject in recent years. In 1943 Li *et al.* and Sayers *et al.* isolated from sheep and ox pituitaries respectively proteins which were homogeneous by sedimentation, electrophoretic and solubility studies. The physicochemical properties of the two substances were similar and the molecular weight of both compounds was approximately 20 000; at that time the substances were regarded as pure proteins. Subsequently, however, certain investigators including Li (1949), Payne *et al.* (1950) and Morris and Morris (1950) were able to prepare from animal pituitaries material of considerably higher biological activity than these two proteins. In view of this finding it appeared justifiable to conclude that the true hormone was probably not a protein but had the composition of a polypeptide having a very much lower molecular weight than was previously supposed. Recently Harris and Li (1954) have shown that sheep ACTH is a polypeptide with thirty nine amino acid residues and with a molecular weight of approximately 4 500; in a subsequent communication Li *et al.* (1955) claim to have elucidated the complete amino acid sequence for this hormone. Bell and his associates (Bell 1954, Howard *et al.*, 1955) have conducted an extensive study into the structure of corticotrophin derived from hog pituitaries. These workers were also able to determine the entire amino acid sequence for this substance

and found that its chemical properties were similar to those reported by Li *et al* (1955) for sheep ACTH

It should be emphasised that, at the time of writing, little or no information is available regarding the chemical nature of the corticotrophin in *human* pituitary tissue

## 2 The Number of Adrenocorticotrophic Hormones

Stack Dunne and Young (1951) assayed pituitary ACTH in hypophysectomised rats by two different methods, one depending on adrenal weight repair and the other on adrenal ascorbic acid depletion They found that the ratio of activities as determined by the two tests varied greatly in different preparations studied It was suggested that the results obtained were in keeping with the view that the pituitary elaborates two adrenocorticotrophic hormones which were designated respectively the adrenal weight factor (AWF) and the ascorbic acid factor (AAF) The views of Stack Dunne and Young (1951) have been challenged by Astwood *et al* (1952) and by others who believe that the differences in biological activity can be explained by differing rates of absorption of ACTH from the site of injection It is clear that further work on the chemical and biological properties of ACTH is necessary before any definite statement can be made on this controversial subject

## 3 ACTH and Intermedin

Within the past few years there has been considerable interest in the possible relationship between these two hormones Intermedin, which is also known as the *melanophore expanding hormone* or *B hormone* is responsible for the colour changes in amphibia The substance is also present in human and mammalian pituitary tissue but its physiological function in man and in mammals remains obscure In 1952 Johnssen and Hogberg presented evidence which appeared to indicate that, in human subjects ACTH and intermedin might be identical or that intermedin might form a part of the ACTH molecule These workers using the method of assay depending on expansion of melanophores in the frog *Rana temporaria* found that the serum of patients with Addison s disease contained large quantities of intermedin and put forward the interesting suggestion that the pigmentation in cases of this

condition might result from excessive production of intermedin by the pituitary Soon afterwards Sulman (1952) and Thing (1952, 1954) in independent investigations claimed to have demonstrated a definite correlation between the content of ACTH and of intermedin in both crude and highly purified ACTH preparations

These observations were obviously of great practical significance in relation to the assay of ACTH in clinical conditions in man for, if ACTH and intermedin were indeed identical, it might then be justifiable to substitute the simple and rapid melanophore expanding test in frogs for tedious and laborious methods employing hypophysectomised rats There are however certain grave objections to the hypothesis that the two hormones are one and the same substance For example, it has been shown (Waring and Landgrebe, 1950) that the biological activity of intermedin is increased by heating with 0 1 N NaOH while that of ACTH is rapidly destroyed by this reagent even at room temperature Furthermore, it is possible to obtain intermedin free of ACTH activity by using the carbon adsorption process described by Landgrebe *et al* (1944) After a survey of the available evidence Morris (1952) concluded that the two hormones were not identical and that the melanophore expanding effects of ACTH were entirely due to contamination However in more recent work Bell (1954) has reached the opposite conclusion In the opinion of this worker it is most unlikely that the melanophore expanding activity of ACTH is due to an impurity From these divergent views it is apparent that the exact relationship of the two hormones one to another is not yet clear and that the problem remains one for future elucidation

## 4 The International Standard for ACTH

The first international standard for ACTH was established by the World Health Organisation in 1950 The material used for the standard was a preparation termed 'La 1 A', this material had already been employed in many centres as a reference standard for the comparative assay of the hormone The international unit was defined as the activity contained in 1 mg of the international standard Results of ACTH assays should now be expressed in international units, 'animal units' should no longer be employed

## METHODS OF ASSAY OF ACTH

Chemical procedures for estimating ACTH in body fluids and in tissues are not yet available and accordingly all assays must be conducted by biological methods Most of the bio assay techniques which have been described are not sufficiently sensitive to detect the presence of the hormone in blood and urine and at the time of writing little or no reliable quantitative information is available regarding the ACTH levels in body fluids in health and disease More reliable assay methods are urgently required, and it is certain that when such techniques are developed and applied in clinical studies much useful information will be obtained

ACTH assays can be conducted in *intact* or in *hypophysectomised* animals Some of the methods which have been proposed are shown in Tables VII and VIII

In general assays in hypophysectomised animals are to be preferred because they are more precise and more specific After hypophysectomy the source of the animal s own secretion of ACTH is removed and the effects produced result entirely from the administered material Sayers (1955) considers that bio assays conducted in animals with intact pituitaries have little or no value

### 1 The Assay of ACTH in Hypophysectomised Animals

The following methods will be considered

(*a*) Adrenal ascorbic acid depletion test in rats
(*b*) Adrenal repair test in rats
(*c*) Adrenal maintenance test in rats
(*d*) Test depending on corticosteroid release in dogs

(*a*) Adrenal Ascorbic Acid Depletion Test (Sayers test) —This is the most important and most widely used assay method for ACTH It depends on the observation that a single dose of ACTH causes a prompt fall in the concentration of adrenal ascorbic acid in hypophysectomised rats As described by Sayers *et al* (1948) the assay is a three day procedure Hypophysectomy is performed on the first day Approximately twenty four hours later the left adrenal is removed and immediately afterwards the animal receives an intravenous injection of standard ACTH or of the test preparation One

hour later the right adrenal is removed On the third day the ascorbic acid concentration of both adrenals is determined using the method of Roe and Kuether (1943) which measures

TABLE VII

METHODS OF ASSAY OF ACTH IN HYPOPHYSECTOMISED ANIMALS

| Animal | Index of Response | Reference |
|---|---|---|
| Rat | Adrenal ascorbic acid depletion (Sayers test) | Sayers *et al* (1948) |
| Rat | Redistribution of adrenal lipoids (repair test) | Simpson *et al* (1943) |
| Rat | Maintenance of adrenal weight (maintenance test) | Simpson *et al* (1943) |
| Mouse | Eosinophil depression | Speirs (1953) |
| Dog | Secretion of corticosteroids | Nelson and Hume (1954) |

TABLE VIII

METHODS OF ASSAY OF ACTH IN INTACT ANIMALS

| Animal | Index of Response | Reference |
|---|---|---|
| Rat | Adrenal weight | Moon (1937) |
| Chick | Adrenal weight | Bates *et al* (1940) |
| Rat | Thymus weight | Bruce *et al* (195 ) |
| Rat | Corticosteroid production by isolated adrenals | Saffran and Bayliss (1953) |
| Rat | Adrenal ascorbic acid depletion in animals pre treated by DCA | Buttle and Hodges (1953) |
| Mouse | Hyaluronidase inhibition | Opsahl and Long (1951) |
| Mouse | Ulcer healing | Clayton and Prunty (1951) |
| Mouse | Eosinophil depression | Speirs (1953) |
| Man | Eosinophil depression | Thorn *et al* (1948) |
| Man | Urinary steroid excretion | Mason *et al* (1948) |

total (reduced plus dehydro) ascorbic acid The response is expressed as the difference between the ascorbic acid content of the right and left adrenal glands

Various modifications of the original Sayers test have been developed One of these substitutes subcutaneous for intravenous administration of the standard and unknown

preparations In the modification described by Munson (1948) both adrenals are removed simultaneously one hour after the intravenous administration of the hormone, and in that of Taylor *et al* (1953) the time interval between hypophysectomy and the commencement of the assay proper is increased from twenty four to forty eight hours

The Sayers test is the most sensitive method at present available for the assay of ACTH and for this reason the technique has been employed in most attempts to estimate the hormone in blood The method will detect as little as 0 2 milliunits of the international standard preparation The technique appears to be satisfactory on grounds of specificity in view of the fact that no other substance has yet been demonstrated which consistently causes a fall in the concentration of adrenal ascorbic acid in hypophysectomised rats The precision of the Sayers test has varied considerably in the hands of different workers (Table IX) Sayers *et al* (1948) found that the index of precision (λ) was generally below 0 2 and concluded that the assay was sufficiently accurate for quantitative work, other investigators including Greenspan *et al* (1950) and Buttle and Hodges (1953), have failed to obtain such a high degree of precision and were unable to use the method on a quantitative basis Morris (1951) has wisely emphasised the importance of the strain of rat in the performance of the Sayers test Some strains appear to be quite unsuitable due to such factors as insensitivity and great variability of response from one animal to another

(*b*) Adrenal Repair Test —The predominant histological change in the adrenal cortex of the rat following hypophysectomy is the disappearance of most of the lipoid from the cells This test which was developed by Simpson *et al* (1943) depends on the ability of ACTH to cause reappearance and redistribution of lipoid in the adrenals of these animals The originators state that the test is satisfactory on grounds of specificity and sensitivity but that it has a low degree of precision The assay method takes nineteen days to perform and in its present form is therefore much too laborious for use in the clinical field

(*c*) Adrenal Maintenance Test —This method, which was also developed by Simpson *et al* (1943) depends on the fact that ACTH maintains normal adrenal weight when

injections of the hormone are started immediately after hypophysectomy The technique is relatively insensitive and requires a two week injection period For these reasons the maintenance test is unsuitable for the estimation of ACTH in human blood and urine

(*d*) TEST DEPENDING ON CORTICOSTEROID RELEASE IN DOGS — Nelson and Hume (1954) have recently described a technique which is sufficiently sensitive to detect very small quantities of ACTH in blood Hypophysectomised dogs are maintained on ACTH until some sixteen hours before the start of the assay At that time the adrenal vein is cannulated by a technique which permits repeated sampling of adrenal venous blood Standard ACTH and the test preparations are administered by *intravenous injection* The end point of the assay is the release of corticosteroids the latter being measured by a *chemical method* depending on the Porter Silber reaction (see p 259)

This technique appears promising as a means of estimating ACTH in blood under normal and pathological conditions Its further application to clinical problems will be awaited with interest

## 2 The Assay of ACTH in Intact Animals

The following methods will be considered

(*a*) Thymus weight test in rats
(*b*) Adrenal ascorbic acid depletion test in rats pre treated by deoxycorticosterone acetate (DCA)
(*c*) Ulcer healing test in mice
(*d*) Hyaluronidase inhibition test in mice
(*e*) Eosinophil depression test in mice
(*f*) Test depending on corticosteroid production by isolated rat adrenals

(*a*) THYMUS WEIGHT TEST (Bruce *et al* 1952) —The basis of this test is the involution of the thymus in infantile intact rats following the injection of ACTH in an oil beeswax medium A three day injection period is required The method is convenient, precise and reasonably specific but is many times less sensitive than the Sayers test

(*b*) *ADRENAL ASCORBIC ACID DEPLETION TEST IN RATS* PRE TREATED BY DEOXYCORTICOSTERONE ACETATE —This test

was introduced by Buttle and Hodges (1953) Hypophysectomised animals were replaced by animals in which the secretion of endogenous ACTH had been inhibited by the administration of DCA In the hands of the originator the method had a low degree of precision (Table IX) The reliability of the technique depends on the degree of pituitary inhibition produced, it is probable that the test is less specific than the original Sayers procedure and should not replace it in quantitative studies

(*c*) ULCER HEALING TEST (Clayton and Prunty 1951) — The end point of this method is the inhibition by ACTH of the formation of experimentally produced granulation tissue in the anterior abdominal wall of the intact mouse The technique is considerably less sensitive than the Sayers test

(*d*) HYALURONIDASE INHIBITION TEST —This method (Opsahl and Long 1951) depends on the ability of ACTH to inhibit the increased capillary permeability induced by hyaluronidase In its present form the test cannot be used for quantitative work Furthermore it is probable that its specificity is questionable

(*e*) EOSINOPHIL DEPRESSION TEST —This technique (Speirs 1953) is based on the fact that ACTH will cause a decrease in the circulating eosinophils in both intact and hypophysectomised mice The method has a relatively low degree of precision (Table IX) and is some fifty times less sensitive than the Sayers test The test therefore cannot be recommended for use in the clinical field

(*f*) TEST DEPENDING ON CORTICOSTEROID PRODUCTION BY ISOLATED ADRENALS —ACTH will stimulate the production of corticosteroids by isolated rat adrenal glands and this has been made the basis of an *in vitro* bio assay method by Saffran and Bayliss (1953) Good agreement is reported between this method and the Sayers test The procedure is reasonably precise and is relatively simple to perform its sensitivity is only slightly inferior to that of the original Sayers test Further observations with this technique especially in relation to the assay of ACTH in body fluids will be awaited with considerable interest

In Table IX the precision of some of the assay methods for ACTH is compared in terms of Gaddum s $\lambda$

TABLE IX

PRECISION OF SOME ASSAY METHODS FOR ACTH

| Method | Animal | Index of Precision λ | Reference |
|---|---|---|---|
| Sayers test | Hypophysectomised rat | 0 176 | Sayers *et al* (1948) |
| Sayers test | Hypophysectomised rat | 0 499 | Greenspan *et al* (1950) |
| Sayers test | Hypophysectomised rat | 0 220 | Taylor *et al* (1953) |
| Sayers test | DCA treated rat | 0 463 | Buttle and Hodges (1953) |
| Adrenal maintenance test | Hypophysectomised rat | 0 376 | Simpson *et al* (1943) |
| Thymus weight test | Intact rat | 0 316 | Bruce *et al* (1952) |
| Eosinophil depression test | Intact mouse | 0 330 | Speirs (1953) |
| Corticosteroid production test | *In vitro* assay in rats | 0 150 | Saffran and Schally (1955) |

## METHODS OF EXTRACTION OF ACTH FROM BLOOD

The quantity of ACTH in human blood is very small, and it is therefore necessary to concentrate the blood prior to bio assay The claims made by certain investigators that ACTH activity can be detected in the blood of normal individuals by injecting untreated serum or plasma into hypophysectomised rats have not been subsequently confirmed

Two main extraction methods have been proposed These are

1 The acid acetone method
2 The oxycellulose method

### 1 The Acid acetone Method

This technique depends on fractional acetone precipitation and was originally developed by Lyons (1937) for the preparation of ACTH from animal pituitaries Various workers including Cooke *et al* (1948), Bornstein and Trewhella (1950) and Gray and Parrott (1953) have used the procedure to extract the hormone from blood Heparinised plasma is precipitated with 80 per cent acetone, the precipitate is

discarded and the acetone concentration of the supernatant fluid is raised to 94 per cent A fine precipitate forms This is centrifuged, separated from the supernatant fluid and dried *in vacuo*

Views are conflicting regarding the efficacy of the acid acetone method According to Parrott (1955) the technique will recover approximately 80 per cent of the activity of Armour s ACTH added to human plasma However, in the experience of Sayers and his co workers (Sydnor *et al*, 1953 *a*) the procedure is very unsatisfactory from the quantitative point of view and frequently yields extracts which are toxic to the experimental animals Sayers (1955) has stated that in his opinion the acid acetone method should no longer be used for the extraction of ACTH from blood

## 2 The Oxycellulose Method

This technique was introduced by Astwood *et al* (1951) for the extraction of ACTH from pituitary tissue and was adapted for estimations in blood by Sydnor and Sayers (1952) The hormone is precipitated by glacial acetic acid is adsorbed on oxycellulose and is eluted from the oxycellulose by 0 1 N NaOH

The oxycellulose technique recovers quantitatively ACTH added to hypophysectomised rat blood and yields concentrates which are non toxic to the experimental animals In the opinion of Sydnor *et al* (1953 *a*) it is the method of choice for the preparation of ACTH from blood

## THE ESTIMATION OF ACTH IN THE BLOOD OF NORMAL SUBJECTS

In assays conducted on human blood the Sayers test due to its high degree of sensitivity has been the bio assay method of choice There is considerable disagreement among various investigators regarding the normal levels of blood ACTH and the results obtained have been both variable and contradictory The experimental evidence has recently been critically examined by Sayers (1955) and the present account is based largely on his conclusions

The most careful and detailed studies of the ACTH content of normal blood have been made by workers at the Mayo Clinic and at Cleveland under the leadership of Albert and Sayers respectively In 1949 Taylor, Albert and Sprague, using

unextracted serum, were unable to demonstrate corticotrophic activity in the blood of normal individuals Subsequently Paris *et al* (1954) extended these observations by employing the oxycellulose extraction method and found that no significant depletion in adrenal ascorbic acid occurred when concentrates equivalent to 40 ml of whole blood were administered to hypophysectomised rats These workers concluded that the concentration of ACTH in normal subjects was very low indeed being usually less than 0 5 milliunits per 100 ml of blood A similar conclusion was reached by Sydnor *et al* (1953 *a*) who used the same methods of extraction and bio assay as those employed by Paris and his co workers

These results are in marked contrast to those reported by Bornstein and Trewhella (1950), Parrott (1951) and Bornstein *et al* (1952), who claimed that corticotrophin was present in normal blood in the very high concentration of 30 to 200 milliunits per 100 ml These workers extracted ACTH from plasma by the acid acetone method, they stated that the hormone was rapidly inactivated in human plasma probably by enzymatic means and that the time elapsing between withdrawal of the blood from the patient and its extraction was critical Other investigators have not confirmed this observation The acid acetone method as used by these investigators did not effect any concentration of the plasma extract and, if the high concentrations of ACTH in plasma reported by Bornstein and his co workers are the true values it is difficult to explain why comparable results could not be obtained by the use of untreated serum

In the opinion of Sayers (1955) and also of the author (Loraine, 1957), the report of very high blood ACTH levels in normal subjects should be viewed with some scepticism It is almost certain that if the concentration of the hormone was indeed 30 to 200 milliunits per 100 ml of blood such levels would most probably be associated with the clinical features of adrenocortical hyperfunction

## BLOOD ACTH IN PATHOLOGICAL CONDITIONS

### 1. Adrenal Hypofunction

All investigators agree that in cases of untreated *Addison's disease* the ACTH titre in blood is increased Taylor *et al*

(1949) were the first to detect activity in the serum of patients with this disease and Sayers (1956) has reported concentrations of 2 to 4 milliunits per 100 ml Treatment of such cases with cortisone causes a decrease in blood ACTH levels, and soon after the start of such therapy activity can no longer be detected in blood

According to Sayers (1955) blood ACTH concentrations rise after *bilateral adrenalectomy* Further observations in patients treated in this way will be awaited with interest

**2 Adrenal Hyperfunction**

Sydnor *et al* (1953 *b*) found abnormally high titres in cases of *congenital adrenal hyperplasia* but Taylor *et al* (1949) were unable to detect any activity in the serum of six patients with *Cushing s syndrome*

**3 'Stress' Conditions**

Under such circumstances elevated blood levels of ACTH are by no means invariable Sayers (1955) found that the administration of concentrates equivalent to 20 to 40 ml of blood obtained from two patients with miliary tuberculosis did not produce any significant depletion of adrenal ascorbic acid in hypophysectomised rats Negative results were also found in healthy male subjects exposed to excessive heat and to abnormally low atmospheric pressure

It must be emphasised that present methods for the deter mination of ACTH in blood are quite unsuitable for routine use in the clinical field Such techniques are expensive and laborious while large volumes of blood are required for individual estimations Nevertheless in centres in which facilities are adequate valuable clinical information can sometimes be obtained in selected cases by the assay of this hormone in blood

## THE ESTIMATION OF ACTH IN URINE

At the time of writing opinions differ as to whether it is possible to estimate ACTH in human urine According to de Barbieri and his co workers (de Barbieri and Zamboni 1953, de Barbieri *et al*, 1953) and to Rubin *et al* (1954)

activity can be detected in both normal and pathological conditions The latter group of investigators, using unextracted urine, found that in normal male subjects the titre ranged from 1 to 3 1 1 u per litre, while in normal females the concentrations lay between 1 and 8 2 1 u per litre In one patient with Cushing s syndrome the ACTH excretion was many times higher than that found in normal individuals On the other hand Sayers *et al* (1949) believe that renal excretion is of little importance as a method of elimination of ACTH These workers administered large single doses of the hormone to healthy volunteers by slow intravenous infusion They were unable to detect ACTH activity in urine either before or after the injection

## THE ESTIMATION OF ACTH IN HUMAN PITUITARY TISSUE

Taylor *et al* (1953) have studied the ACTH concentration in lyophilised pituitary tissue from adults, infants and fœtuses In the adult and infantile glands the ACTH content expressed in terms of dried weight ranged from 49 to 588 1 u per g Corticotrophic activity was detected in fœtal pituitaries at a period of gestation as early as sixteen weeks In the infantile glands where in all cases the death autopsy interval was less than twenty four hours there was no apparent relationship between this interval on the one hand and the ACTH content of the pituitary on the other The number of cases studied was too small to permit of any definite conclusion regarding the stability of ACTH in the pituitary after death However, destruction of the hormone did not appear to be unduly rapid

## THE ESTIMATION OF ACTH IN HUMAN PLACENTAL TISSUE

Various authors including Jailer and Knowlton (1950), Opsahl and Long (1951) and Cohen and Kleinberg (1952) have reported that ACTH is present in extracts prepared from human placental tissue Cohen and Kleinberg (1952) extracted placentæ by the oxycellulose method and found a concentration of ACTH of 4 to 5 1 u per kg fresh tissue Additional observations on the placental concentration of ACTH in

normal and pathological conditions will be anticipated with interest

## Summary and Conclusions

ACTH assays should be conducted in hypophysectomised rather than intact animals All results should be expressed in terms of the international standard preparation The most promising techniques from the point of view of their application to clinical problems are those depending on adrenal ascorbic acid depletion in hypophysectomised rats (Sayers test), on the production of corticosteroids by isolated rat adrenals and on the release of corticosteroids in hypophysectomised dogs

ACTH can be extracted from blood by methods involving acid acetone precipitation or oxycellulose adsorption

The concentration of ACTH in the blood of normal individuals is very low being usually less than 0 5 milliunits per 100 ml of blood Increased titres have been reported in Addison s disease in congenital adrenal hyperplasia and following bilateral adrenalectomy

Opinions differ as to whether it is possible to demonstrate the presence of ACTH in human urine

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## CHAPTER VI

# *Growth Hormone*

## INTRODUCTION

THIS hormone is secreted by the anterior pituitary and is responsible for skeletal and visceral growth Other names for growth hormone are *somatotrophin* and the *somatotrophic hormone* (STH)

### 1 Chemical Nature of Growth Hormone

In 1945 Li *et al* claimed to have isolated growth hormone in pure form from extracts of ox pituitary glands The hormone was found to be a protein with a molecular weight of 44 250 It behaved as a homogeneous substance in electrophoretic, diffusion and solubility studies It was relatively insoluble in water and had an iso electric *p*H of 6 85 Growth promoting activity was destroyed by treatment with pepsin and trypsin, free amino group were considered necessary for the biological activity of the hormone

Purified preparations of growth hormone from animal pituitaries have also been made by Wilhelmi *et al* (1948) These workers used a method of extraction similar to that employed by Cohn *et al* (1946) in fractionating blood proteins This method involved alcohol precipitation at low temperatures The chief advantages of Wilhelmi s technique are its relative ease of performance and the high yields of the hormone obtained However it was found that the crystals so prepared exhibited two components on electrophoretic examination and accordingly it must be concluded that the preparation was less pure than that of Li *et al* (1945)

It should be emphasised that at present little or no information is available regarding the chemical properties of growth hormone in *human* pituitary tissue

### 2 Growth Hormone and Diabetogenic Hormone

Many investigators now hold the view that the diabetogenic hormone of the anterior pituitary does not exist as a separate

entity and that this effect is mediated by growth hormone by ACTH or by the two hormones acting in conjunction It has, for example, been shown that highly purified preparations of growth hormone made by the method of Li *et al* (1945) are actively diabetogenic in intact cats Young (1941, 1945) believes that a close relationship exists between the growth promoting activity and diabetogenic actions of anterior pituitary extracts This worker found that prolonged administration of actively diabetogenic extracts to puppy dogs initially causes acceleration of growth uncomplicated by diabetes mellitus If however, the treatment is continued for several months the animals cease to grow and a diabetic condition supervenes Young's observations are obviously of great importance in relation to the development of diabetes mellitus in man However, the exact role played by growth hormone in the ætiology of human diabetes is at present not clear, and the problem remains one for future elucidation

At present no *international standard* is available for the comparative assay of growth hormone In view of the large number of 'animal units' currently in use in bio assays of this substance, the establishment of such a standard preparation would appear to be highly desirable

## METHODS OF ASSAY OF GROWTH HORMONE

A large number of methods have been suggested for the bio assay of growth hormone Many of these are reasonably satisfactory for estimations in pituitary tissue, but none of them is sufficiently sensitive to detect the presence of the hormone with any regularity in human blood or urine The development of an assay method for growth hormone which would be suitable for use in clinical studies is one of the most urgent requirements in present day endocrinology

Some of the assay methods proposed are shown in Table X which is taken from an article by Li (1953) This worker has suggested that the available techniques should be divided into two groups, the first consisting of reasonably well established procedures, and the second of methods which have not yet gained general acceptance or have so far been incompletely investigated Only the techniques in the first group will be considered in this chapter

TABLE X

METHODS AVAILABLE FOR THE BIO ASSAY OF GROWTH HORMONE

(*After Li* 1953)

**A Well-established Procedures**

1 Increase in weight in normal plateaued rats
2 Increase in weight in hypophysectomised rats
3 Increase in weight in dwarf mice
4 Increase in tail length in hypophysectomised rats
5 Increase in width of the proximal epiphyseal cartilage of the tibia in hypophysectomised rats

**B Suggested Procedures**

1 Increase in liver weight
2 Increase in weight in stilbœstrol treated rats
3 Changes in the nitrogenous constituents of the blood
4 Changes in serum phosphorus or phosphatase
5 Changes in the nitrogen or phosphorus balance
6 Increase in protein synthesis as demonstrated by radio active techniques

## 1 Increase in Weight in Normal Plateaued Rats

In this test which was originally described by Evans and Simpson (1931) six month old female rats are employed Such animals normally gain weight very slowly and are spoken of as plateaued rats Intraperitoneal injections of growth hormone will cause these animals to resume growth, as evidenced by gain in weight

As shown by Fønss Bech (1947) and by Greenspan *et al* (1950) this method is reasonably satisfactory with regard to precision and specificity Its chief disadvantages lie in its high degree of insensitivity and in the laborious nature of the procedure which requires an injection period extending over fifteen to twenty days It is obvious that the method in its present form is quite unsuitable for use in the clinical field

## 2 Increase in Weight in Hypophysectomised Rats

In the technique described by Marx *et al* (1942) immature female rats are hypophysectomised at an age of approximately

entity and that this effect is mediated by growth hormone, by ACTH or by the two hormones acting in conjunction It has, for example, been shown that highly purified preparations of growth hormone made by the method of Li *et al* (1945) are actively diabetogenic in intact cats Young (1941, 1945) believes that a close relationship exists between the growth promoting activity and diabetogenic actions of anterior pituitary extracts This worker found that prolonged administration of actively diabetogenic extracts to puppy dogs initially causes acceleration of growth uncomplicated by diabetes mellitus If, however, the treatment is continued for several months the animals cease to grow and a diabetic condition supervenes Young s observations are obviously of great importance in relation to the development of diabetes mellitus in man However, the exact role played by growth hormone in the ætiology of human diabetes is at present not clear, and the problem remains one for future elucidation

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## 2 Increase in Weight in Hypophysectomised Rats

In the technique described by Marx *et al* (1942) immature female rats are hypophysectomised at an age of approximately

four weeks and are rested for ten to twelve days post operatively The injection period has varied from ten to fifteen days in the hands of different investigators

According to Greenspan *et al* (1950) this test, although rather less precise, is some thirty times more sensitive than the method employing plateaued rats However, the technique is still not sufficiently sensitive for use in clinical studies

## 3 Increase in Weight in Dwarf Mice

Details of this method have been given by Fønss Bech (1947) and by Kemp (1948) These workers used a strain of mice showing hereditary dwarfism Such animals gain weight under treatment with growth hormone provided that injections are continued for two to three weeks

According to Greenspan *et al* (1950) this technique has a relatively low degree of precision and is of doubtful specificity The chief advantage of the test is the fact that intact rather than hypophysectomised animals can be used

## 4 Increase in Tail Length in Hypophysectomised Rats

In the procedure described by Dingemanse *et al* (1948) male rats aged six to eight weeks are used The injections commence immediately after hypophysectomy and are continued for a period ranging from one to two weeks This test is relatively insensitive and has a low degree of precision Furthermore its specificity is questionable in view of the fact that other hormones notably TSH and thyroid hormone are capable of influencing the response

## 5 Tibial Test in Hypophysectomised Rats

This method (Evans *et al* 1943) depends on the ability of growth hormone to increase the width of the proximal epiphyseal cartilage of the tibia in hypophysectomised rats The chief advantage of this test is its relatively high degree of sensitivity Greenspan *et al* (1950) have shown that the tibial test is some ten to twenty times more sensitive than that depending on weight gain in hypophysectomised rats A further advantage is the short injection period of only four days

Recent evidence presented by Li (1953) suggests that the

tibial test is not so specific as was previously supposed This worker found that thyroid hormone, TSH and ACTH were all capable of interfering with the response, and suggested that ideally assays should be conducted in animals which had been subjected to hypophysectomy, adrenalectomy and thyroidectomy

## Comparison of the Precision of Assay Methods for Growth Hormone

In Table XI, which is taken from an article by Greenspan *et al* (1950) the precision of various assay methods for growth hormone is compared in terms of Gaddum's λ

TABLE XI

PRECISION OF SOME ASSAY METHODS FOR GROWTH HORMONE

(*After Greenspan et al* 1950)

| Method | Index of Precision λ | Reference |
|---|---|---|
| Plateaued rat weight test | 0 198 | Marx *et al* (1942) |
| | 0 226 | Fønss Bech (1947) |
| Hypophysectomised rat weight test | 0 265 | Marx *et al* (1942) |
| | 0 354 | Bulbring (1938) |
| | 0 402 | Fønss Bech (1947) |
| Dwarf mice weight test | 0 34 | Fønss Bech (1947) |
| | 0 670 | Fønss Bech (1947) |
| Tail length test in hypophysectomised rats | 0 532 | Fønss Bech (1947) |
| Tibial test in hypophysectomised rats | 0 225 | Dingemanse *et al* (1948) |
| | 0 310 | Greenspan *et al* (1949) |
| | 0 330 | Gemzell and Heijkenskjold (1956) |

It will be noted that a reasonable degree of precision can be expected with the plateaued rat weight test with the tibial test and to a less extent with the hypophysectomised rat weight test On the other hand methods depending on the increase in weight in dwarf mice and on the increase in tail length in hypophysectomised rats appear to lack precision and should probably not be used in quantitative work

## METHODS OF EXTRACTION OF GROWTH HORMONE FROM BODY FLUIDS

### 1 Urine

Growth promoting activity has not yet been detected in human urine under normal or pathological conditions There is at present no information available on what type of method should be used for the preparation of the hormone from this source

### 2 Blood

Kinsell *et al* (1948) injected the equivalent of from 3 to 10 ml of lyophilised plasma from a patient with gigantism into hypophysectomised female rats and found that the tibial epiphysis in these animals was appreciably wider than that of either uninjected controls or of hypophysectomised rats injected with lyophilised plasma from normal subjects Greenspan (1950) was unable to demonstrate growth promoting activity in pooled lyophilised plasma collected from a large number of growing children

Gemzell *et al* (1955) added pituitary growth hormone to human plasma and extracted the plasma by a method similar to that used by Cohn *et al* (1946) for the fractionation of plasma proteins Bio assays were conducted using the tibial test in hypophysectomised rats The added growth hormone was detected in Cohn s fractions IV 1 and IV 4 The total yield of the hormone was approximately 50 per cent

## CLINICAL APPLICATIONS OF GROWTH HORMONE ASSAY

Growth hormone has not yet been detected in the blood of normal individuals but growth promoting activity has been found in the plasma of a small number of patients with gigantism and acromegaly (Kinsell *et al*, 1948, Gemzell *et al*, 1955) Recently Gemzell *et al* (1955), using the tibial test in hypophysectomised rats, have succeeded in demonstrating the presence of the hormone in lyophilised plasma from pooled retroplacental blood and in plasma from umbilical cord blood Further work along these lines will be awaited with interest

As stated previously, a method for the quantitative determination of growth hormone in body fluids is urgently required,

and when developed such a technique would have wide applications in clinical research Two of the conditions in which growth hormone assays might be especially informative are *pregnancy complicated by diabetes* and *malignant disease*

In diabetic pregnancy the birth weight of the baby is frequently greater than normal The reason for this abnormality is not known with certainty, but the suggestion has been made that it results from, or is associated with, the presence of excessive quantities of circulating maternal growth hormone (Gray and Feemster, 1926 Barns and Morgans, 1948) If reliable assays of this hormone could be performed it might then be possible to establish whether a correlation does indeed exist between the maternal growth hormone levels on the one hand and the size of the baby on the other

Widespread interest has been aroused by the claims of Moon and his collaborators (Moon *et al* 1950, 1951) that malignant tumours tend to develop in intact female rats injected with growth hormone over long periods of time and that such tumours do not usually occur in hypophysectomised rats so treated It is not yet known whether growth hormone plays any part in carcinogenesis in humans Further information on this important subject must await the development of more sensitive assay methods for this hormone in body fluids

## Summary and Conclusions

Assay methods for growth hormone are not yet sufficiently sensitive for general application in clinical studies The most promising technique is probably that depending on the increase in width of the proximal epiphyseal cartilage of the tibia in hypophysectomised rats

Further work is necessary on methods of extraction of growth hormone from blood and from urine

Growth promoting activity has been demonstrated in the blood of patients with acromegaly and gigantism, in pooled retroplacental blood and in umbilical cord blood Activity has not yet been found in the blood of normal subjects

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## METHODS OF EXTRACTION OF GROWTH HORMONE FROM BODY FLUIDS

### 1 Urine

Growth promoting activity has not yet been detected in human urine under normal or pathological conditions There is at present no information available on what type of method should be used for the preparation of the hormone from this source

### 2 Blood

Kinsell *et al* (1948) injected the equivalent of from 3 to 10 ml of lyophilised plasma from a patient with gigantism into hypophysectomised female rats and found that the tibial epiphysis in these animals was appreciably wider than that of either uninjected controls or of hypophysectomised rats injected with lyophilised plasma from normal subjects Greenspan (1950) was unable to demonstrate growth promoting activity in pooled lyophilised plasma collected from a large number of growing children

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## CLINICAL APPLICATIONS OF GROWTH HORMONE ASSAY

Growth hormone has not yet been detected in the blood of normal individuals but growth promoting activity has been found in the plasma of a small number of patients with gigantism and acromegaly (Kinsell *et al*, 1948, Gemzell *et al*, 1955) Recently Gemzell *et al* (1955), using the tibial test in hypophysectomised rats have succeeded in demonstrating the presence of the hormone in lyophilised plasma from pooled retroplacental blood and in plasma from umbilical cord blood Further work along these lines will be awaited with interest

As stated previously, a method for the quantitative determination of growth hormone in body fluids is urgently required,

# CHAPTER VII

## *Prolactin*

### INTRODUCTION

THIS hormone is elaborated by the anterior lobe of the pituitary and is essential for the initiation and probably also for the maintenance of lactation in man and animals Other names for prolactin are the *lactogenic hormone galactin* and *mammotrophin* Many workers believe that prolactin is identical with the gonadotrophic factor *luteotrophin*

### 1 Chemical Nature of Prolactin

Purified preparations of prolactin were first made by Lyons (1937) from sheep pituitary tissue Subsequently Li *et al* (1940) showed that sheep prolactin behaved as a pure protein in electrophoretic and solubility studies The molecular weight of the hormone was estimated to be approximately 25,000 and its iso electric *p*H was 5 5

### 2 The "Mammogen" Theory

There has been considerable controversy regarding the mechanism whereby the ovarian hormones cause mammary growth and development Turner and his associates (Gomez *et al* 1937 Gomez and Turner 1938) have claimed that in animals œstrogens and progesterone stimulate the pituitary to secrete specific hormones which they have termed 'mammogens and which are believed to act directly on the breast tissue Other investigators have been unable to confirm these observations The evidence for and against the mammogen theory has recently been critically reviewed by Folley (1955) who concluded that in the rat there was no necessity to postulate the existence of specific mammogenic hormones It is probable that a similar conclusion is justifiable in other species including man

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## 1 Assays Employing Pigeons

Prolactin preparations stimulate the crop glands of pigeons by causing rapid proliferation of the epithelial lining In addition the hormone increases the production of ' crop milk which is a caseous fluid consisting mainly of desquamated epithelial cells Assay methods in pigeons are generally more sensitive and more precise than those in mammals but require rigid standardisation of technique during their performance Among the many factors which have been shown to influence the response are the season of the year the body weight of the birds, the strain and race of pigeon, the environmental temperature the route of injection and the volume of solution administered

Three variants of the crop gland test have been described These are

(*a*) The crop weight method (Riddle *et al* 1933)
(*b*) The minimum stimulation method (McShan and Turner, 1936)
(*c*) The local intradermal method (Lyons and Page, 1935)

(*a*) The Crop Weight Method —In the technique described by Riddle *et al* (1933) the birds are injected intramuscularly daily for four days and are killed on the fifth day Riddle and Bates (1939) have shown that there is a linear relationship between the crop weight on the one hand and the logarithm of the dose of prolactin on the other Coppedge and Segaloff (1951) have used this technique to estimate the prolactin activity of human urine They claimed that the sensitivity of the assay could be increased by using the intravenous rather than the intramuscular route of injection and by administering a small booster dose of prolactin to all groups of birds under test In the experience of Clarke and Folley (1953) the crop weight test was reasonably precise the index of precision (λ) was generally less than 0 2 when ten birds were used on each dose level of standard and unknown preparations

(*b*) The Minimum Stimulation Method —In this procedure the birds are injected subcutaneously instead of intramuscularly After ninety six hours the crop glands are removed and merely examined against the light for a positive reaction A positive effect is indicated by the presence of typical strands

### 3 Prolactin and Luteotrophin

It is now widely believed that the gonadotrophic hormone luteotrophin is identical with prolactin Luteotrophin maintains the activity of the corpus luteum in the non pregnant animal and is also responsible for the secretion of progesterone by the ovary Its physiological function in man remains obscure

As pointed out by Astwood (1953) the evidence for the identity of prolactin and luteotrophin is suggestive but not entirely conclusive The strongest support for the view comes from the observation that prolactin preparations, purified to the extent of behaving as homogeneous proteins in electrophoretic and solubility studies, are highly active both in initiating milk secretion and in causing corpus luteum maintenance However, recent experience with other pituitary hormones, notably ACTH and growth hormone, has demonstrated that such criteria of purity should not be regarded as final Further work is necessary in order to ascertain whether a similar conclusion should also be drawn in the case of prolactin

### 4 The International Standard for Prolactin

Prolactin was the first pituitary hormone for which an international standard was prepared This was established at the Third International Conference on the Standardisation of Hormones (1938) By definition 1 i u is the activity contained in 0 1 mg of the standard preparation All assay results should now be expressed in terms of this material and not in ' animal units

## METHODS OF ASSAY OF PROLACTIN

Many bio assay methods for prolactin have been proposed but the majority of these are too insensitive for clinical use Assays can be divided into the following two groups

1 Assays employing pigeons
2 Assays employing mammals

For details regarding the actual technique and design of prolactin assays the reader is referred to articles by Li and Evans (1948) Meites and Turner (1950) and Turner (1950)

This test is easy to perform but its success appears to depend to a large extent on the strain of animal employed The originators have not claimed that the method is specific for prolactin In view of the fact that positive responses are elicited in the test animals with total doses of urine as small as 2 ml it would appear unlikely that the technique measures prolactin activity alone

(*c*) ASSAYS IN RABBITS —Bradley and Clarke (1956) have recently described an assay method for prolactin which appears promising for the estimation of this hormone in body fluids The end point of the assay depends on milk formation in the mammary glands of rabbits after the intraductal injection of prolactin Œstrogen progesterone treated pseudo pregnant or pregnant animals may be used Intraductal injection of pituitary extracts rich in activities other than prolactin does not cause milk formation and accordingly it can be stated that this method is highly specific for the lactogenic hormone In its present form this test has a relatively low degree of precision and it is probable that minor modifications in design are necessary before the assay can be adapted for quantitative work

## METHODS OF EXTRACTION OF PROLACTIN FROM BODY FLUIDS

It has not yet been possible to demonstrate with certainty the presence of prolactin in *blood* but claims have been made that the hormone is present in extracts prepared from *urine* The method of extraction generally employed is similar to that described by Klinefelter *et al* (1943) for the preparation of pituitary gonadotrophins from urine and depends on alcohol or acetone precipitation with or without dialysis (see pp 29 and 30) According to the available evidence the yield of prolactin obtained by this technique is not high For example Coppedge and Segaloff (1951), who performed recovery experiments by adding pituitary prolactin to pooled urine found that more than 50 per cent of the biological activity was lost during the extraction procedure In view of this observation it is doubtful whether the results reported for the excretion of prolactin in normal and pathological conditions in man have much significance from the quantitative point of view

of thickened mucosa The procedure in its present form is purely qualitative and cannot be used for quantitative work

(c) THE LOCAL INTRADERMAL METHOD —This is a microtechnique in which the solutions under test are injected immediately over the crop gland Epithelial proliferation associated with basophilic changes in the cytoplasm of the cells forms the end point of the assay The method is claimed to be many times more sensitive than that depending on crop weight and has been used for the assay of prolactin in human urine According to Bahn and Bates (1956) the method is highly specific for prolactin provided that basophilic changes in the cytoplasm rather than mere epithelial thickening is made the end point of the test Little is at present known regarding the precision of the test but it is probable that the error is relatively large

## 2 Assays Employing Mammals

Prolactin can be assayed by methods depending on the *induction of lactation in guinea pigs* Such techniques are at present too insensitive for clinical use and will not be considered further Some comment is, however, necessary on mammalian assays involving rats, mice and rabbits

(a) ASSAYS IN RATS —Tests for luteotrophic activity in rats have been reviewed by Astwood (1953) Methods have depended on the prolongation of the œstrus cycle in intact rats, on the inhibition of the œstrus vaginal smear in œstrogen treated hypophysectomised rats and on the formation of deciduomata in the uteri of hypophysectomised pseudopregnant rats None of these tests has yet been applied with any success to the assay of luteotrophic activity in the body fluids of humans

(b) ASSAYS IN MICE —Hadfield and his co workers (Scowen and Hadfield, 1955, Hadfield 1956, Hadfield and Young 1956) have recently shown that urine extracts from normally menstruating and post menopausal women are capable of stimulating the proliferation of mammary epithelium in weanling male mice and have suggested that this effect might be made the basis of an assay method for determining the 'mammotrophic' activity of human urine The end point of the assay depends on the enumeration of the deeply staining terminal 'clubs which are formed at the growing ends of the ducts, each club consists of a mass of undifferentiated mammary epithelium

this disease They were unable to demonstrate any relationship between the prolactin output on the one hand and the response of the patient to various forms of treatment on the other Hadfield (1956) has recently suggested that ' mammotrophin assays may be helpful in patients in whom hypophysectomy is being considered as a therapeutic measure This interesting suggestion requires further study

## Summary and Conclusions

Assay methods for prolactin are not yet sufficiently sensitive for routine use in the clinical field Among the more promising techniques are those depending on the crop weight in pigeons and on milk formation in the mammary glands of rabbits

Further work is necessary on methods of extraction of prolactin from body fluids

Claims have been made that prolactin activity is present in human urine under both normal and pathological conditions but it is doubtful whether the results reported so far have much quantitative significance

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## THE CLINICAL APPLICATION OF URINARY PROLACTIN ASSAYS

### 1 Normal Subjects

At the time of writing there is much conflict of opinion regarding the amounts of prolactin excreted in human urine Early work by Lyons and Page (1935), by Meites and Turner (1941) and others suggested that the hormone could be detected in the urine of lactating women but not under other conditions In a recent study Bahn and Bates (1956), using the very sensitive local intradermal method in pigeons, were unable to detect the presence of prolactin in urine in normal subjects of both sexes These workers concluded that, if the hormone was excreted in urine at all, the amounts present were less than 5 i u per twenty four hours

The results of Bahn and Bates (1956) are in marked contrast to those reported by Coppedge and Segaloff (1951) using the crop weight method in pigeons and by Hadfield (1957) using the mammary proliferation test in mice Coppedge and Segaloff (1951) stated that in normal men and in normally menstruating women the urinary output of prolactin ranged from 0 to 300 i u per twenty four hours and that, in women in late pregnancy, the excretion values varied from 50 to 300 i u per twenty four hours Hadfield (1957) has recently reported that urine obtained from pre menopausal women *contains concentrations of prolactin ranging from 10 to 250* i u per litre

It seems reasonable to conclude that these divergent findings can be explained on a methodological basis In view of the high degree of specificity of the technique employed by Bahn and Bates (1956) it is probable that the figures reported by these authors more closely approximate the 'true' values The assay methods used by Coppedge and Segaloff (1951) and by Hadfield (1957) are of doubtful reliability and it is not unlikely that such techniques provide an over estimate of the true potency and thus yield erroneously high urine values

### 2 Pathological Conditions

MAMMARY CARCINOMA —Segaloff and his co workers (Segaloff, 1953, Segaloff *et al* 1954) have attempted to estimate the urinary excretion of prolactin in patients with

## CHAPTER VIII

# *Antidiuretic Hormone and Antidiuretic Substances*

## INTRODUCTION

THE term *antidiuretic hormone* (ADH) should be reserved for the substance which is liberated by the posterior lobe of the pituitary and which controls water excretion by acting on the cells of the renal tubules The term *antidiuretic substance* (ADS) refers to any substance or group of substances

TABLE XII

PRINCIPAL PHARMACOLOGICAL EFFECTS OF THE POSTERIOR PITUITARY HORMONES

(*After Van Dyke* 1955)

| Effect on | Hormone | |
|---|---|---|
| | Oxytocin | Vasopressin |
| Water diuresis | No effect | Inhibits |
| Blood pressure | Slightly lowers | Raises |
| Coronary arteries | Slightly dilates | Constricts |
| Intestinal contractions | Slightly stimulates | Stimulates |
| Uterine contractions | Stimulates | Stimulates |
| Ejection of milk | Stimulates | Slightly stimulates |

The response varies according to many factors including the species and the stage of the reproductive cycle

present in serum plasma or tissue extracts which are capable of causing antidiuresis when administered to hydrated animals Serum, plasma or tissue extracts with this property are said to possess *antidiuretic activity* (ADA) *Vasopressin* and *oxytocin* are two highly active polypeptides which are secreted by the posterior pituitary Their principal pharmacological properties are shown in Table XII Van Dyke and his co-workers (Van Dyke 1955, Van Dyke *et al*, 1955) believe that vasopressin

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TABLE XIII

ASSAY METHODS FOR POSTERIOR PITUITARY ANTIDIURETIC ACTIVITY

*(After Heller and Blackmore 1952 and Dicker 1953)*

| Test Animal | Mode of Administration of Test Solutions | Water Load | Anæsthesia | Sensitivity | Reference |
|---|---|---|---|---|---|
| Dog (intact) | Subcutaneous | 200 ml per animal by mouth | None | 0 25 to 0 5 milliunit per animal | Kestranek *et al* (1925) |
| | Intravenous | Not stated | None | 0 5 milliunit per animal | Van Dyke *et al* (1955) |
| Dog (with experimental *diabetes insipidus*) | Intravenous | 100 ml per animal by mouth | None | 0 2 to 0 3 milliunit per animal | Hare *et al* (1945) |
| Rat | Subcutaneous | 5 ml per 100 g by mouth | None | 2 to 3 milliunits per 100 g | Burn (1931) |
| | Intraperitoneal | 2 to 5 ml per 100 g by mouth followed by 5 ml per 100 g | None | 0 5 to 1 milliunit per 100 g | Ham and Landis (1942) |
| | Intravenous | 5 ml 12 per cent alcohol per 100 g by mouth followed by 3 ml water per 100 g by mouth | Alcohol | 0 02 to 0 03 milliunit per animal | Jeffers *et al* (194 ) |
| Rabbit | Intravenous | 100 ml by mouth followed by [illegible]0 ml per kg | Paraldehyde | 0 5 milliunit per animal | Walker (1939) |
| Mouse | Intraperitoneal | [illegible] ml per 100 g intraperitoneally | None | 0 milliunits per 10 g | Nelson and Woods (1934) |
| | Intravenous | Three times 5 ml per 100 g orally | None | 0 010 milliunit per 10 g | Heller and Blackmore (1952) |

is identical with the antidiuretic hormone The term *pitressin* is not synonymous with either ADH or with vasopressin although the pharmacological activity of pitressin is probably due to its vasopressin content

**1 Chemical Nature of Oxytocin and Vasopressin**

The structures of both compounds have been recently elucidated through the work of investigators in New York Vienna and Paris (Livermore and du Vigneaud, 1949, Turner *et al*, 1951, Acher *et al*, 1952, Tuppy, 1953) Both substances are polypeptides containing eight amino acids Oxytocin has the same structure in both hog and ox pituitary tissue, *in ox vasopressin the amino acid arginine* replaces the lysine present in hog vasopressin The molecular weight of oxytocin is 1 007 while that of ox vasopressin is 1,084 Du Vigneaud and his colleagues (du Vigneaud *et al*, 1953, 1954) finally succeeded in synthesising both oxytocin and vasopressin They found that the natural and synthetically prepared products were identical in all respects

**2 The International Standard for Posterior Pituitary Hormones**

This was established in 1936 by the Commission on Biological Standardisation of the Health Organisation of the League of Nations The standard was prepared by extraction of ox posterior lobes with acetone followed by drying of the insoluble residue The international unit is the activity present in 0 5 mg of the international standard This amount of material contains by definition one unit of oxytocic activity, one unit of pressor activity and one unit of antidiuretic activity

## METHODS FOR THE ESTIMATION OF ANTIDIURETIC ACTIVITY

This subject has been reviewed by Thorp (1950), Pickford (1952), Heller and Blackmore (1952), Van Dyke *et al* (1955) and others All the methods depend on the administration of standard and unknown preparations to animals which are in a state of water diuresis In general rats or dogs have been the test animals of choice in such assays Table XIII shows some of the methods proposed along with their approximate sensitivities

ensure a satisfactory degree of precision the test should be repeated in two or three days time, using a cross over design in which the animals which were previously injected with the standard are injected with the unknown and those previously receiving the unknown are given the standard

Burn s method with minor modifications in design has been widely used in attempts to estimate the ADS present in the blood and urine of man and animals Gilman and Goodman (1937) found that a more consistent response was obtained after the administration of a preliminary hydrating dose of water while Ginsburg (1951) improved the sensitivity of the method by administering the water in three doses at hourly intervals In the techniques described by Ham and Landis (1942) and by Birnie *et al* (1949) the assay was made more sensitive by increasing the water load and by substituting intraperitoneal for subcutaneous injection of the standard and unknown preparations For details of the actual procedures recommended the reader is referred to the original articles

Probably the most reliable method in rats for the estimation of the antidiuretic activity of body fluids is the intravenous technique described originally by Jeffers *et al* (1942) and subsequently modified by Ames and Van Dyke (1952) and by Dicker (1953) The assay animals are rendered diuretic by the administration of water by gavage They are also given ethanol by the same route this produces light anæsthesia and probably also suppresses the secretion of endogenous ADH Urine is collected by means of catheters inserted into the bladder When the urine excretion is relatively constant the standard or unknown preparation is injected intravenously Estimations of antidiuretic activity are made by comparing the effect of the unknown with the standard in the same animal The technique is very sensitive and will detect as little as 0 02 milliunit of the standard Recently Hawker (1956 *a b*) has used this method to study the antidiuretic activity of serum plasma and placental extracts under normal and pathological conditions in man His results will be discussed later in this chapter

## 2 Methods employing Dogs

Assays in intact dogs have been described by various investigators including Hare *et al* (1941), O Connor (1950)

Many of the procedures listed in Table XIII are reasonably satisfactory for the estimation of the relatively large quantities of ADH present in posterior pituitary tissue. It is, however, doubtful whether any of them, with the possible exception of the intravenous techniques, are sufficiently specific to permit of the quantitative determination of this hormone in the body fluids of man and animals. It is well known that blood and urine contain many pharmacologically active substances other than ADH which are capable of causing antidiuresis in hydrated rats. For example, Erspamer and Sala (1954) have suggested that the antidiuretic activity of rabbit serum results from its content of 5 hydroxytryptamine (5HT). It must also be borne in mind that the subcutaneous and intraperitoneal injection of the test solutions is liable to cause the animals pain and excitement, both of which stimuli *per se* can produce antidiuresis by promoting the secretion of endogenous ADH.

Fluids suspected of containing ADS have been administered to test animals by the subcutaneous, intraperitoneal and intravenous routes. Although vasopressin itself can be conveniently assayed when injected by all three routes, most workers now agree that the ADS present in blood and urine can be estimated with reasonable accuracy only when the material containing it is given intravenously (Heller, 1951, Ames and Van Dyke, 1952, Pickford, 1952, Van Dyke *et al.*, 1955).

## 1 Methods employing Rats

The method described by Burn (1931) depends on observing the degree of antidiuresis produced when standard and unknown preparations are administered subcutaneously to groups of hydrated rats. Adult male animals are employed, they are starved for some twelve hours prior to the assay but are allowed free access to water during this period. Each animal receives 5 ml of water per 100 g body weight by gavage and immediately afterwards the test solutions are injected. Urine volumes are measured at fifteen minute intervals for approximately three hours. The time elapsing between the injection of the extracts and the point of maximum diuresis is noted and is plotted against the logarithm of the dose of the standard preparation. The potency of the unknown is determined by reference to the dose response curve for the standard. To

to be conducted, the blood should be concentrated prior to bio-assay

Various techniques have been recommended for the preparation of ADS from *urine* In that of Noble *et al* (1939) the material is adsorbed on zinc ferricyanide and is then eluted by 1 per cent ammonia in alcohol The ammonia and alcohol are removed by vacuum distillation and the dry residue is dissolved in water The originators claim that, when posterior pituitary extract is added to urine and extracted in this way 70 to 90 per cent of the antidiuretic activity is recovered

Ralli *et al* (1945) and others have extracted antidiuretic substances from urine merely by evaporating the urine at room temperature to volumes of approximately 80 ml The reduction in volume is completed in thirty six to forty eight hours and during this time the electrolytes are removed by dialysis The final solution is made up to 100 ml with water One millilitre of this solution is administered to each of the test animals Slessor (1951) has employed the ultrafiltration method of Gorbman (1945) for the preparation of ADS from urine Details of this technique are given on page 35

## THE CLINICAL APPLICATIONS OF ASSAYS OF ADH AND ADS

ADH estimations in the clinical field will become possible only when assay methods more specific for this hormone are developed Attempts have been made by various investigators to determine the concentration of ADS in body fluids under normal and pathological conditions but because of the unreliable nature of the assay methods used, it is doubtful whether any confidence can be placed in the results obtained Some of the problems encountered in such estimations have been critically examined by Lewis (1953) This worker compared the antidiuretic activity of human serum when assayed by two methods one involving rats and the other man Normal volunteers were subjected to dehydration for twelve hours after this period the serum was assayed by intraperitoneal injection into hydrated rats and was shown to possess considerable antidiuretic activity The potency

and Van Dyke *et al* (1955) In the opinion of Van Dyke *et al* (1955) the most satisfactory test animal for the assay of ADH is the heavily hydrated, unanæsthetised dog which has been trained to lie quietly in a supine position throughout the experiment Water is administered to such animals by gavage and, when the excretion of urine has become relatively constant, the standard and unknown preparations are injected intravenously The end point of the assay is the degree of antidiuresis produced The test is reasonably precise and is probably more specific for ADH than any other method yet proposed It is also very sensitive and will detect as little as 0 25 milliunit of the standard preparation Adamsons *et al* (1956) have used this test to study the ADH content of pituitary glands in camels in order to ascertain whether or not the hormone has a special role in water conservation in this animal It is probable that, in the future, the technique will be the procedure of choice for the quantitative determination of ADH in the body fluids of human subjects

Hare *et al* (1945) have recommended the use of dogs with experimentally produced diabetes insipidus for the assay of ADH Theoretically assays conducted in such animals should be more reliable as the endogenous production of the hormone will not interfere with the results However, Van Dyke *et al* (1955) state that in normal dogs which have been adequately trained prior to the assay the secretion of ADH from the posterior pituitary is insufficient to influence the results obtained These workers concluded that assays conducted in trained animals with intact pituitaries were just as satisfactory as those performed in dogs with experimental diabetes insipidus

## THE EXTRACTION OF ADH AND ADS FROM BODY FLUIDS

At the time of writing little or no reliable information is available as to what method or methods should be used for the extraction of ADH and ADS from *blood* Up till now most investigators have administered untreated serum or plasma to the test animals As pointed out by Lewis (1953) and by others it is probable that even in dehydrated subjects the blood concentration of ADH (as distinct from ADS) is very low Accordingly, if assays specific for this hormone are

that in such individuals the liver had lost its capacity to inactivate ADH Hall *et al* (1949) and Dochios and Dreifus (1951) have also reported an increase in antidiuretic activity in the urine of subjects with portal cirrhosis Labby (1949) found an abnormally high urinary excretion of ADS in cases of *infective hepatitis*, the titre decreased during the period of convalescence from the disease

In a more recent study Perry and Fyles (1953) estimated the concentration of ADS in serum in a series of nine patients with liver disease They found normal values in all cases and concluded that alterations in the level of ADS in serum were not responsible for the water retention associated with hepatic cirrhosis

(c) MISCELLANEOUS DISEASES —Assays of ADS in blood and urine have been performed in numerous other clinical conditions, a few of which will be briefly mentioned In *congestive cardiac failure* with œdema and in the *nephrotic syndrome* various investigators have demonstrated the presence of increased antidiuretic activity in urine (Robinson and Farr, 1940, Bercu *et al*, 1949, Dochios and Dreifus 1951) Claims have been made that a positive correlation exists between the quantity of ADS excreted on the one hand and the degree of clinical œdema on the other These observations are at variance with the findings of Perry and Fyles (1953) who showed that the levels of ADS in the serum of ten patients with congestive cardiac failure did not differ significantly from normal

It is well known that in *Addison s disease* and in other types of adrenal insufficiency water metabolism is abnormal The failure of prompt diuresis after the ingestion of water forms the basis of the Robinson Power Kepler test which has been widely used in the diagnosis of patients with adrenal insufficiency Slessor (1951) has compared the antidiuretic activity of body fluids in patients with Addison s disease with that in normal individuals Untreated serum and urine extracted by the ultrafiltration method were injected intraperitoneally into hydrated rats Individuals in both groups were subjected to a period of fourteen hours dehydration It was found that at the end of this time the serum concentration of ADS was much higher in the Addisonians than in the control subjects The antidiuretic activity of urine was also determined

was equivalent to approximately 2 milliunits of pitressin (Parke Davis) per millilitre Following dehydration serum was also reinjected into the donors No antidiuresis was observed and it was calculated that the activity present was less than 0 1 milliunit of pitressin per millilitre From this discrepancy in results Lewis (1953) concluded that the rat assay method was non specific and that the antidiuretic effect produced in the test animals resulted either from a stimulus such as pain or from the presence in serum of substances other than ADH

## 1 Assays of ADS in Non-pregnant Conditions

(a) NORMAL SUBJECTS —Various workers, including Lloyd and Lobotsky (1950), Hawker (1953) and Perry and Fyles (1953) have demonstrated the presence of ADS in the serum of normal men and women Lloyd and Lobotsky (1950) and Perry and Fyles (1953) have also reported that the concentration of ADS in serum rises after dehydration and falls during the diuresis induced by the ingestion of water In these investigations the intraperitoneal assay method in rats as described by Birnie *et al* (1949) was employed In a recent study Hawker (1956 *a*), using the more specific intravenous technique of Jeffers *et al* (1942), was unable to demonstrate antidiuretic activity in either serum or plasma from normal subjects *Hawker s findings provide support for the view that* assays conducted by methods depending on intraperitoneal injection give an over estimate of true antidiuretic potency and thus yield erroneously high values

(*b*) LIVER DISEASE —Evidence is conflicting regarding the concentration of ADS in the body fluids of patients with *hepatic cirrhosis* Ralli *et al* (1945) compared the antidiuretic activity of normal urine with that of cirrhotic patients both with and without ascites The urine was concentrated by evaporation and was assayed in rats by Burn s method The antidiuretic activity of normal urine and of the urine of cirrhotic patients without ascites was relatively low In cirrhotic subjects with ascites, on the other hand, abnormally high levels were found Ralli *et al* (1945) suggested that the antidiuretic substance found in urine might be derived from the posterior pituitary and that the reason for its demonstration in increased amounts in the urine of cases of cirrhosis was due to the fact

inactivate oxytocin and vasopressin the reader is referred to articles by Werle and Effkemann (1941) and by Page (1946)

(*b*) PRE ECLAMPTIC TOXÆMIA —It is not yet known with certainty whether the excessive secretion of ADH plays any part in the ætiology of this condition The information obtained from assays of ADS in body fluids has been confusing and contradictory Anselmino *et al* (1932) claimed to have demonstrated an increase in ADS in the blood of eclamptic women and suggested that the material was derived from the posterior pituitary Teel and Reid (1939) reported that urine concentrates from toxæmic patients showed greater amounts of antidiuretic activity than those of normal women they also stated that the quantity of antidiuretic material could be correlated with the degree of water retention but not with the hypertension or albuminuria On the other hand, various investigators, including Theobold (1934) Levitt (1936) and Krieger and Kilvington (1946), were unable to demonstrate the presence of abnormally large quantities of ADS in the body fluids of toxæmic patients

The subject of antidiuretic substances in body fluids in normal and abnormal pregnancy has recently been reviewed by Pickford (1952) This worker concluded that at present there was no definite evidence to support the view that the over production of pituitary ADH with associated water retention was the main cause of pre eclamptic toxæmia It remains to be established whether antidiuretic substances are indeed present in increased quantities in the blood and urine of these patients or whether their apparent detection is due to the use of unsuitable assay methods

(*c*) PLACENTAL EXTRACTS —Hawker (1956 *b*) has recently shown that saline extracts of placentæ from normally pregnant women and from patients with pre eclamptic toxæmia are capable of inactivating both the oxytocic and antidiuretic activity of posterior pituitary extracts He has suggested that the inhibitor present in placental tissue is an enzyme Placentæ from normally pregnant women were found to possess considerably more enzymic activity than those from toxæmic women Hawker s observations are certainly of interest in relation to the ætiology of pre eclamptic toxæmia but their exact significance remains a problem for future elucidation

in both groups after the administration of a test dose of water In the normal subjects maximum diuresis occurred during the second hour of the test, at this time the urine, when tested for antidiuretic activity, gave negative results In the Addisonian patients, on the other hand, water diuresis was either absent or greatly delayed Urinary assays for ADS conducted during the first four hours after the administration of water showed definite activity whereas urine passed during the period of delayed diuresis produced no response in the test animals On the basis of these observations Slessor (1951) concluded that the presence of an antidiuretic substance in the blood of patients with Addison's disease was responsible for the delayed diuresis seen in their condition He also suggested that, since the phase of delayed diuresis is associated with the absence of antidiuretic activity from urine, the rate of removal of the ADS from blood in Addison's disease is abnormally slow It must be emphasised that, although these suggestions are of interest, they must be accepted with reserve in view of the relative non specificity of the assay method used in the course of this investigation

## 2 Assays of ADS during Pregnancy

(*a*) Normal Pregnancy —Various workers, including Teel and Reid (1939) and Krieger and Kilvington (1946), have failed to demonstrate the presence of antidiuretic activity in the blood and urine of normally pregnant women On the other hand Hawker (1953) using the assay method of Birnie *et al* (1949), detected ADS in the serum throughout normal pregnancy The levels remained relatively constant at all stages of pregnancy but appeared to fall just prior to the onset of lactation In more recent studies with a more specific assay method Hawker (1956 *a*) found antidiuretic activity in the serum but not in the plasma of normally pregnant women at different stages of gestation He also showed that normal pregnancy plasma contains a substance which inactivates both vasopressin and the ADS present in serum from normally pregnant women and in plasma from cases of pre eclamptic toxæmia The properties of this inhibitor in pregnancy plasma are at present unknown although observations by Hawker (1956 *b*) and by others have suggested that it may be enzymic in nature For further information regarding the ability of pregnancy blood to

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## SUMMARY AND CONCLUSIONS

Assay methods for ADH are not yet sufficiently specific for routine application in the clinical field The most promising techniques at present available are those employing intravenous injection of the test solutions into hydrated rats and dogs Assays depending on subcutaneous and intraperitoneal administration probably give an over estimate of antidiuretic potency and should no longer be used in clinical studies

Further work is necessary on methods of extraction of ADH from blood and urine

It has been stated that the antidiuretic activity of body fluids is increased in hepatic cirrhosis with ascites, in the nephrotic syndrome, in congestive cardiac failure with œdema, in Addison s disease and in pre eclamptic toxæmia These claims should be accepted with reserve in view of the unreliability of the assay methods employed

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## CHAPTER IX

# Œstrogens

## INTRODUCTION

FIVE different, but closely related, œstrogens have so far been isolated from human urine These substances are *œstradiol 17β*, *œstrone œstriol*, 16 *epiœstriol* and 16*α hydroxy œstrone* The first three œstrogens have been known for many years while the fourth and fifth have only recently been shown to be naturally occurring compounds The structural formulæ of the various œstrogens are shown in Fig 32

From the chemical point of view the three main characteristics of the œstrogens are (1) the aromatic nature of ring A of the steroid nucleus, (2) the oxygen substituent at C 17 and (3) the phenolic OH group at C 3 which gives the compounds weakly acidic properties

*Œstradiol 17β* was originally isolated by MacCorquodale *et al* (1935) from the follicular fluid of sow s ovaries and has subsequently been isolated from other sources including human pregnancy urine (Huffman *et al* 1940 *a*) human placental tissue (Huffman *et al*, 1940 *b*), horse testes (Beall, 1940) and stallion urine (Levin 1945) This substance is the most potent of the naturally occurring œstrogens as judged by bio assays in rats and mice and, because of this fact the suggestion has been made that œstradiol 17β is the true ovarian hormone in man

*Œstrone* has been isolated from many sources including human pregnancy urine (Doisy *et al* 1929) human male urine (Dingemanse *et al*, 1938) human placental tissue (Westerfeld *et al*, 1938) beef adrenal glands (Beall, 1939) and the bile of pregnant cows (Pearlman *et al*, 1947) As judged by bio assays in rats and mice, œstrone is less active than œstradiol 17β In chemical structure it differs from the latter in having an oxo (=O) group instead of a hydroxyl (–OH) group at C 17

*Œstriol* has been isolated from human pregnancy urine

(Marrian, 1930) and from human placental tissue (Browne, 1931) The presence of the hormone has not yet been detected with certainty in the urine of other species In bio-assays involving oophorectomised rats and mice, œstriol is less active in causing vaginal cornification than are œstradiol 17$\beta$ or œstrone The molecule of œstriol contains three hydroxyl groups and this accounts for the greater water solubility of the compound

A stereo isomer of œstriol 16 *epiœstriol* has recently been

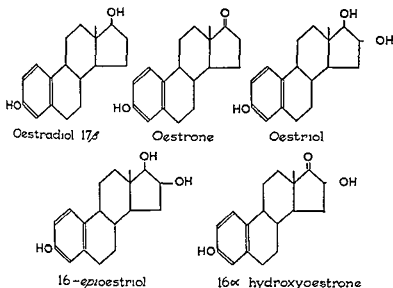



Fig 32
Structural formulæ of the various œstrogens

isolated from human pregnancy urine (Marrian and Bauld 1955) Various workers including Huffman and Grollman (1947) and Loraine *et al* (1957) have demonstrated that this substance is also capable of causing vaginal cornification in oophorectomised rats and mice but that its biological activity in this test is considerably less than that of œstriol

Marrian *et al* (1957) have recently succeeded in isolating from human pregnancy urine a fifth œstrogen, 16$\alpha$ *hydroxyœstrone* When assayed by the vaginal smear test in oophorectomised mice 16$\alpha$ hydroxyœstrone and œstriol are approximately equipotent (Loraine *et al*, 1957)

Œstrogens are excreted in human urine as water soluble conjugates Œstriol is conjugated with glucuronic acid as œstriol glucuronide It was formerly believed that œstrone was probably conjugated with sulphuric acid and excreted as œstrone sulphate However, recent work by Oneson and Cohen (1952) has indicated that a relatively large proportion of the œstrone in human pregnancy urine is conjugated as the glucuronide The mode of excretion of œstradiol 17β, 16 *epi*œstriol and 16α hydroxyœstrone is not yet known

It is almost certain that human urine contains unknown œstrogen metabolites in addition to the five known compounds The evidence for this statement is based mainly on radioactivity studies and will be considered later in this chapter (p 172)

## SOURCES OF ŒSTROGENS IN THE BODY

The main sources of œstrogen production in humans are the *ovary* and the *placenta* Œstrogens are also secreted in smaller amounts by the *adrenal cortex* and by the *testis*

### 1 Ovary

Most workers now hold the view that the main site of œstrogen production in the human ovary is the theca interna rather than the stratum granulosum Œstrogens are secreted both by the maturing Graafian follicle and by the corpus luteum after ovulation

Some doubt still exists as to the nature of the œstrogens secreted by the human ovary Many years ago MacCorquodale *et al* (1935, 1936) isolated œstradiol 17β from the follicular fluid of sow's ovaries and in 1938 Westerfeld *et al* demonstrated the presence of œstrone in the same material Since then it has been generally assumed that these two substances are secreted by the ovaries of other species including man It should however, be emphasised that the œstrogens produced in and secreted by the human ovary have not yet been identified with certainty, although there is some indirect evidence, obtained from studies of the œstrogen excretion during the normal menstrual cycle, that œstradiol 17β and/or œstrone may be the principal ovarian œstrogens elaborated in humans

**2 Placenta**

Œstrogens were first demonstrated in placental tissue by Fellner in 1912 Subsequently œstriol, œstrone and œstradiol 17β were isolated from human placental tissue Various workers including Diczfalusy (1953) Mitchell and Davies (1954) and Diczfalusy and Lindkvist (1956) have shown that, of the placental œstrogens, œstriol is the most important from the quantitative point of view

There is good evidence to support the view that during pregnancy the placenta takes over from the ovary the production of œstrogens and that from the second or third month of pregnancy onwards this organ is the dominant source of the hormone

**3 Adrenal Cortex**

Œstrone has been isolated from beef adrenal glands and, although œstrogens have not yet been identified with certainty in human adrenal tissue there is strong indirect evidence to support the view that the adrenal cortex is capable of elaborating these substances Various investigators have detected small quantities of œstriol, œstrone and œstradiol 17β in the urine of normal men, and of post menopausal and oophorectomised women and it is probable that the principal source of œstrogens in such individuals is the adrenal cortex Both male and female patients with certain types of adrenocortical tumours have been shown to excrete abnormally large quantities of œstrogens in their urine (Simpson and Joll 1938, Dohan *et al*, 1953) and it is reasonable to assume that these substances are derived from hyperactive adrenocortical tissue In addition it has been recently demonstrated by Dao (1953) and by Brown (1957) that in patients with mammary carcinoma subjected both to bilateral oophorectomy and bilateral adrenalectomy the urinary œstrogen titre may fall to zero levels

**4 Testis**

Goldzieher and Roberts (1952) have recently detected the presence of œstradiol 17β in human testicular tissue, while Diczfalusy (1954) has shown that pooled human semen contains œstradiol 17β œstrone and œstriol Little is so far known regarding the site of production of œstrogens in the testis Various investigators including Vigdoff *et al* (1939)

and Berthrong *et al* (1949), have suggested that the seminiferous tubules, and in particular the Sertoli cells, are capable of œstrogen secretion, but the evidence for this is by no means conclusive For further information on this controversial subject the interested reader is referred to review articles by Huggins and Moulder (1945) and by Howard *et al* (1950)

## METHODS OF ESTIMATION OF ŒSTROGENS IN HUMAN URINE

In this section consideration will be given to assay methods for œstrogens in urine extracts At the time of writing, assay methods for these hormones in blood are not in a sufficiently advanced state to merit critical evaluation

As mentioned previously the œstrogens present in the urine do not occur in the free state but are conjugated with glucuronic acid and perhaps also, to a lesser extent, with sulphuric acid Prior to extraction and determination either by biological or by chemical methods, these conjugates must be hydrolysed This may be accomplished either by boiling the urine with acid or by incubating it with enzyme preparations containing β glucuronidase and phenolsulphatase The free œstrogen is then extracted with a suitable water immiscible solvent, *e g*, ether Subsequent washing of the ethereal extract by sodium bicarbonate removes the acid fraction of urine, the total phenolic fraction which can be used for the determination of total œstrogens, can then be obtained by distribution between ether, benzene or toluene and sodium hydroxide Finally, the total phenolic fraction can be separated into œstriol, œstrone and œstradiol fractions and the excretion of the individual œstrogens can then be determined

Most of the early work on the estimation of œstrogens in urine extracts was performed by bio assay techniques which, although sensitive, had a relatively low degree of precision By means of such techniques a considerable amount of useful clinical information was accumulated on the œstrogen excretion in health and disease, but the results obtained could not be regarded as strictly quantitative Within recent years reliable chemical methods have become available for the assay of urinary œstrogens, and it is virtually certain that these techniques will entirely replace bio assay procedures in the estimation of

œstrogens for clinical purposes However, in view of the fact that mo t of the results at present available for the œstrogen excretion in patients have been obtained by bio assay, it was felt that these techniques as well as the chemical procedures should be briefly described

## THE ESTIMATION OF ŒSTROGENS BY BIOLOGICAL METHODS

The discussion will be conducted under the following headings

1 The international standard preparations for the œstrogens
2 Assays based on vaginal cornification
3 Assays based on uterine weight
4 Miscellaneous assay methods

### 1 The International Standard Preparations for the Œstrogens

An international standard for œstrone was established in 1932 by the Permanent Commission on Biological Standardisation The international unit was defined as the activity contained in 0 1 $\mu$g of the standard preparation The introduction of this standard made it possible for workers to assay urine extracts in terms of œstrone and to express results in international units rather than in animal units However for reasons which will be discussed later in this chapter, the establishment of a standard preparation did not greatly increase the reliability of œstrogen assays by biological methods and the clinical information obtained remained of doubtful value from the quantitative point of view

In the years immediately following the adoption of this standard esterified forms of the hormone came into general use When these preparations were tested against the standard for œstrone the relative potency was found to vary depending on the solvent and on the assay design employed Accordingly, in 1935 the Permanent Commission on Biological Standardisation agreed to establish a second international standard namely, the monobenzoate of œstradiol 17$\beta$ The international unit was again defined as the activity contained

in 0 1 $\mu g$ of the standard preparation The standard for œstradiol monobenzoate has not been used to any extent in œstrogen assays in the clinical field

**2 Assays based on Vaginal Cornification**

The biological method for œstrogen assay most frequently employed in clinical studies depends on the induction of vaginal cornification in oophorectomised rats and mice after the subcutaneous administration of urine extracts This technique was originally introduced by Allen and Doisy in 1923 and has since been studied extensively and modified by numerous investigators In the modification described by Marrian and Parkes (1929) aqueous solutions of œstrogens are injected subcutaneously into mice in four doses over thirty six hours Vaginal smears are taken once on the second day, three times on the third day and twice on the fourth day of the assay The response is considered positive if any of the six smears taken from a mouse is positive A 'positive' smear is regarded as one which contains epithelial and cornified cells but no leucocytes Using the vaginal smear test it has generally been found that œstradiol 17$\beta$ is more active than œstrone and that œstrone is more active than œstriol

Many investigators in the clinical field have expressed assay results with the vaginal smear test in 'rat' and mouse units, a unit being defined as the dose of urine necessary to elicit 50 per cent of positive responses in a group of animals It has already been emphasised (p 8) that the error of estimations expressed in animal units may be very large and that results calculated in this way have little quantitative significance

Emmens (1939) has made a careful study of the many variable factors affecting the vaginal smear test He concluded that, for a reasonable degree of precision a four point design should be employed with two groups of mice receiving the standard and two groups receiving the unknown preparation Each group should consist of not fewer than twenty animals Emmens (1939) and others have found that when the number of animals per dose level of standard or unknown is less than twenty the error of the test becomes very large

Various attempts have been made to increase the precision and sensitivity of the Allen Doisy test One of the modifications

suggested substituted for subcutaneous injection intravaginal application of œstrogens (Emmens, 1947) Various workers including Albrieux (1941) and Krichesky and Glass (1947), have applied the intravaginal technique to the assay of œstrogens in human blood

## 3 Assays based on Uterine Weight

Methods have been described using oophorectomised immature rats (Bulbring and Burn, 1935) and intact immature rats and mice (Lauson *et al* 1939, Evans *et al* 1940) Usually the animals are injected once or twice per day for three days and are killed approximately seventy two hours after the first injection at which time the uteri are weighed fresh In general there is a linear relationship between the logarithm of the dose of the administered œstrogen and the effect produced although the actual dose response curves for the various œstrogens are markedly dissimilar Assays in intact immature rats have shown that œstradiol 17$\beta$ is the most potent of the natural œstrogens and that œstriol is more potent than œstrone (Lauson *et al*, 1939) Similar studies in mice by Evans *et al* (1940) and others have demonstrated that œstriol is the weakest of the three œstrogens that œstradiol 17$\beta$ is the most potent, and that œstrone occupies an intermediate position

Six hour Test for Œstrogenic Activity —Astwood (1938) studied the early effects of œstrogen on the uteri of intact immature rats and showed that a rapid increase in weight occurred during the first few hours after the injection This increase is mainly due to accumulation of water by the endo metrial stroma Astwood (1938) proposed that this reaction be made the basis of a six hour assay method in which the dosage of the œstrogens administered was plotted against the percentage increase in uterine weight Using this technique it was found that œstradiol 17$\beta$ was approximately twelve times more potent than œstrone

## 4 Miscellaneous Assay Methods

Various additional bio assay methods for œstrogens have been described These have included tests depending on *vaginal opening* in intact immature rats mice and guinea pigs (Lauson *et al* 1939 Littrell *et al* 1946) and on *enlargement of the oviduct* in chicks (Dorfman and Dorfman, 1948) However

none of these procedures has yet gained popularity in the clinical field For details of these methods the reader is referred to the original articles

## Disadvantages of Biological Methods in the Assay of Urinary Œstrogens

The careful studies of Emmens (1939) and of Pedersen Bjergaard (1939) have demonstrated beyond all doubt that for a number of reasons, bio assays of œstrogens when performed on the relatively impure extracts derived from human urine, are so unreliable that little or no confidence can be placed in the results obtained The sources of error fall into three main groups

In the first place it has been shown that, in a given experiment the estimation of œstrogenic potency will depend to a very large extent on the method of assay adopted and that minor variations in technique such as alteration of solvent or of spacing of injections will markedly influence the final result This is well illustrated by the observation of Pedersen Bjergaard (1939), who found that an extract of human pregnancy urine gave a potency varying from 158 to 75,900 i u per g (expressed in terms of œstrone) according to the assay method employed

The second difficulty arises from the fact that, since the impure urine extracts contain a mixture of at least three œstrogens which differ widely in their biological activity, such extracts cannot be assayed in terms of the international standard for œstrone A more reliable estimate of œstrogenic potency would presumably be obtained by separating the œstrogens prior to bio assay into œstriol, œstrone and œstradiol fractions and by expressing results in terms of the weights of the pure crystalline substances However, such a procedure would be exceedingly laborious and time consuming and would require a very large number of animals for any reasonable degree of precision

A third source of error depends on evidence which has been presented by Emmens (1939) This worker has shown that human urine concentrates may contain augmenting' substances which enhance the effect of œstrogens on the target organs and which are present in varying amounts according to the method of extraction employed

## THE ESTIMATION OF ŒSTROGENS BY CHEMICAL METHODS

Chemical procedures for the determination of œstrogens in human urine fall into two main groups

1 Colorimetric methods
2 Fluorimetric methods

### 1 Colorimetric Methods

Such methods have usually depended on the reaction described originally by Kober in 1931 This test consists essentially of heating the œstrogens with a mixture of phenol and sulphuric acid, diluting with water and re heating A pink colour with an absorption maximum at a wave length of approximately 520 mμ is obtained Later workers have shown that other phenols and some reducing agents can be substituted for phenol itself in the reagent

The Kober reaction is highly specific for the naturally occurring œstrogens Its chief source of error lies in the fact that crude œstrogen concentrates from urine contain substances of unknown composition which produce a brown colour in the spectral range of the absorption maximum of the Kober colour Various attempts have been made to correct for the error in estimation due to the brown colour and thus to increase the sensitivity of the procedure (Venning *et al* 1937 Stevenson and Marrian 1947) Such attempts were reasonably successful in the case of extracts from the urine of pregnant women which contain relatively large amounts of œstrogens but, until recently were much less successful in the case of urinary extracts from non pregnant subjects in whom the excretion of œstrogens is very much lower In 1948 Marrian concluded that the Kober reaction together with the extraction and purification procedures then available was probably unreliable in urines containing less than 2 mg of œstrogens per twenty four hours and that accurate chemical determinations of the urinary excretion of œstrogens were only possible during mid and late pregnancy

Recently careful and detailed studies have been made by Brown (1952) and by Bauld (1954) on the many variable factors concerned in the development of the Kober colour and this work has resulted in a very significant increase in the

reliability of the procedure Great improvements were also made by those investigators in methods of extraction and purification of the urinary œstrogens The methods ultimately developed by Brown (1955 *a*) and by Bauld (1956) for the quantitative determination of œstradiol 17$\beta$, œstrone and œstriol in human urine are described below

(*a*) THE METHOD OF BROWN (1955 *a*) —The main steps in this method are (i) acid hydrolysis, (ii) ether extraction, (iii) carbonate wash of the ether extract (iv) partition between a benzene-petroleum ether mixture and water and alkali, (v) a new phase change purification procedure for the phenolic fraction depending on methylation of the phenol group, (vi) separation of the œstrogen methyl ethers by adsorption chromatography on alumina columns (vii) colorimetric measurement using an improved Kober colour method (viii) spectrophotometric correction for interfering chromogenic material using the formula described by Allen in 1950[1] It should be noted that the œstrogens obtained by this procedure are in the form of methyl ethers and are not biologically active

The accuracy of the method has been tested by a series of recovery experiments in which known amounts of œstriol, œstrone and œstradiol 17$\beta$ were added to portions of acid hydrolysed twenty four hour urine samples from normal men The mean recovery figures lay between 80 and 90 per cent even at levels corresponding to 4 $\mu$g of added œstrogen per twenty four hours It is probable that a further 10 to 20 per cent of the added œstrogens is lost during acid hydrolysis and that the overall recovery for the whole method is of the order of 60 to 75 per cent

In his original paper Brown (1955 *a*) stated that his method was reasonably accurate for the determination of œstradiol 17$\beta$, œstrone and œstriol down to a level of 5 $\mu$g per twenty four hours of each of the three œstrogens Subsequent work has tended to show that although the precision and accuracy of the method decrease when the amount of each œstrogen present is less than 5 $\mu$g per twenty four hours results above

[1] The Allen correction depends on the assumption that the contaminating colours have linear wave length/absorption curves in the region of the absorption maximum of the œstrogen Kober colours There is good evidence to show that this assumption is correct in the case of the methods described by Brown (1955 *a*) and by Bauld (1956)

approximately 2 μg per twenty four hours for each of the three œstrogens still retain quantitative significance (British Empire Cancer Campaign, 1956)

The specificity of Brown s method has been studied by Diczfalusy (1955) using countercurrent techniques and by Bulbrook *et al* (1957) using bio assay There is now good evidence to support the view that the substances measured are indeed œstradiol 17β œstrone and œstriol

This method for œstrogen determination is somewhat exacting and time consuming but is suitable for use in the clinical field One trained technician can conveniently perform four to six complete estimations in two days Various investigators, including Brown (1955 *b* 1956) Diczfalusy and Westman (1956), Bulbrook and Greenwood (1957 *a* *b*) and Støa and Knutsen (1957) have used the procedure to study the urinary excretion of œstrogens in health and disease

(*b*) The Method of Bauld (1956) —This method involves (i) acid hydrolysis (ii) ether extraction (iii) separation of œstriol from œstrone and œstradiol 17β by distribution between benzene and water (iv) purification of œstriol by saponification and by column partition chromatography, (v) separation and purification of œstrone and œstradiol 17β by column partition chromatography and by saponification, (vi) colorimetric determination of the purified fractions using an improved Kober reaction (vii) spectrophotometric correction for interfering chromogenic material by means of Allen s correction formula

Bauld s method differs from that of Brown in employing partition chromatography on celite columns rather than adsorption chromatography on alumina columns The œstrogens obtained by Bauld s method are in the free form and are therefore biologically active

Comparison of Methods of Brown and Bauld —The methods are comparable in terms of accuracy precision sensitivity and specificity When parallel assays by the two methods were conducted on urine samples from men and from normally menstruating and post menopausal women the results obtained were in good agreement (Marrian 1955)

There is little doubt that the methods of Brown and Bauld are the best procedures at present available for the quantitative determination of œstrogens in human urine The main

limitation of both methods lies in the fact that the conditions of hydrolysis are not entirely optimal and it is probable that from 10 to 20 per cent of the œstrogens is lost during this step The methods are well adapted to studies in women during reproductive life but are probably less reliable in post menopausal women, in men and in children, in whom the urinary excretion of œstrogens is generally lower, being frequently less than 5 μg per twenty four hours for each of the three œstrogens Neither method in its present form is capable of measuring the more recently discovered urinary œstrogens, 16 *epi*œstriol and 16α-hydroxyœstrone In order to obtain a true picture of the urinary excretion of endogenous œstrogens in health and disease it will probably be necessary to design a new procedure which will permit of the accurate determination of all five compounds

## 2 Fluorimetric Methods

Such methods depend on the fact that an intense yellowish green fluorescence develops when an œstrogen is heated with sulphuric acid or phosphoric acid The fluorescence produced is proportional to the quantity of œstrogens present, its intensity can be measured in a sensitive fluorimeter The fluorescence reaction for œstrogens is more sensitive than the Kober reaction It has, however the serious disadvantage of being much less specific This lack of specificity arises from the fact that, in the relatively impure extracts prepared from urine by existing methods substances other than œstrogens are capable of producing fluorescence

Numerous methods involving fluorimetry have been described, but for various reasons none of them has gained wide acceptance in clinical studies The method described by Jailer (1948) was applied to the estimation of œstrone and œstradiol 17β in urine but could not be used for the quantitative determination of œstriol A more promising technique was later described by Engel *et al* (1950) These workers purified their extracts by countercurrent distribution and subsequently estimated the individual œstrogens by fluorimetry This technique is much too laborious for routine use This can also be said of a further refinement of Engel s method developed by Migeon (1953) In the method of Braunsberg *et al* (1955) the phenolic material, obtained after hydrolysis

extraction and partial purification of urine, was further purified by partition chromatography on celite columns, fluorimetric estimations of œstrone, œstradiol 17β and œstriol were made on the three fractions thus obtained Very recently Aitken and Preedy (1956) have described an assay procedure for urinary œstrogens involving gradient elution partition chromatography followed by fluorimetry At the time of writing it is too early to assess whether this technique will prove suitable for application to clinical problems

There seems little doubt that at present, colorimetric rather than fluorimetric techniques should be used for the quantitative determination of œstrogens in human urine The many variable factors in the fluorescence reaction have not yet been adequately studied Such a study might well yield important information as this reaction because of its relatively high sensitivity might in the future become the procedure of choice for the final determination of œstrogens *in low titre urines and in blood* This view is supported by the recent work of Preedy and Aitken (1957) who using an assay method depending on gradient elution partition chromatography and fluorimetry, were able to measure the plasma œstrogen concentration in normally pregnant women

## 16 *EPI*ŒSTRIOL AND 16α HYDROXYŒSTRONE AS URINARY ŒSTROGENS

By using the highly specific Kober reaction as modified by Brown and by Bauld Marrian and his co workers have recently detected in human urine the presence of substances other than œstradiol 17β, œstrone and œstriol which are capable of giving the Kober reaction (Marrian and Bauld 1955, Watson and Marrian, 1956 Marrian *et al* 1957)

In 1955 Marrian and Bauld, using pregnancy urine were able to concentrate a new Kober chromogen in the crude œstriol containing fraction Subsequently by means of countercurrent solvent partition methods and column partition chromatography, these workers separated this substance completely from œstriol and isolated it as a pure crystalline compound This compound was shown to be an isomer of œstriol, it was soon demonstrated that it was identical with 16 *epi*œstriol a substance which many years before had

limitation of both methods lies in the fact that the conditions of hydrolysis are not entirely optimal and it is probable that from 10 to 20 per cent of the œstrogens is lost during this step The methods are well adapted to studies in women during reproductive life but are probably less reliable in post menopausal women, in men and in children, in whom the urinary excretion of œstrogens is generally lower, being frequently less than 5 μg per twenty four hours for each of the three œstrogens Neither method in its present form is capable of measuring the more recently discovered urinary œstrogens, 16 *epi*œstriol and 16α hydroxyœstrone In order to obtain a true picture of the urinary excretion of endogenous œstrogens in health and disease it will probably be necessary to design a new procedure which will permit of the accurate determination of all five compounds

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between the slopes of the dose effect curves for the two œstrogens It is therefore justifiable to assay 16 *epi*œstriol in terms of œstriol

Marrian *et al* (1957) have recently succeeded in isolating from pregnancy urine a fifth œstrogen, 16α *hydroxyœstrone* In late pregnancy urine quantities of 16α hydroxyœstrone ranging from 1 to 3 mg per twenty four hours have been found (see p 181), and it is therefore probable that from the quantitative point of view, this steroid is as important as œstrone and more important than œstradiol 17β In urine collected from women during the normal menstrual cycle values ranging from 1 to 25 μg per twenty four hour urine sample have been obtained It must be emphasised that at present methods for the estimation of 16α hydroxyœstrone in urine are far from quantitative For this reason it is probable that the true values for this urinary steroid are much higher than those quoted above

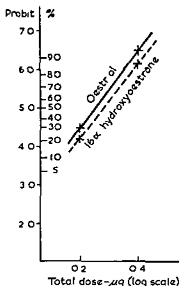



Fig 34

Comparison of the biological activities of 16α hydroxyœstrone and œstriol using the vaginal smear test in oophorectomised mice (*From Loraine et al* 1957)

Loraine *et al* (1957) have shown that œstriol and 16α hydroxyœstrone are approximately equipotent when assayed by the vaginal smear test in oophorectomised mice A typical assay is shown in Fig 34

It will be noted that the slopes of the dose response curves for the two œstrogens do not differ significantly It can therefore be concluded that as in the case of 16 *epi*œstriol it is justifiable to assay 16α hydroxyœstrone in terms of œstriol

## THE CLINICAL SIGNIFICANCE OF URINARY ŒSTROGEN ESTIMATIONS

One of the main reasons for performing urinary œstrogen determinations is to obtain information regarding the

been prepared synthetically but had not previously been shown to occur naturally There is now good evidence that 16 *epi*œstriol occurs as such in urine and is not an artifact produced during the extraction process Watson and Marrian (1956) have recently described a method which permits of the rough determination of 16 *epi*œstriol in pregnancy urine and have also detected the presence of this steroid in urine obtained from women during the follicular and luteal phases

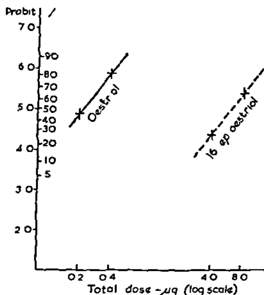



Fig 33

Comparison of the biological activities of 16-*epi*œstriol and œstriol using the vaginal smear test in oophorectomised mice (*From Loraine et al* 1957)

of the normal menstrual cycle In cyclic urine the quantity present was approximately 1 µg per twenty four hour specimen

The œstrogenic potency of 16 *epi*œstriol has been compared with that of œstriol using the vaginal smear test in oöphorectomised rats and mice (Loraine *et al*, 1957) 16-*epi*œstriol was found to be capable of causing vaginal cornification in such animals but was considerably less active than œstriol in this respect A typical assay in mice is shown in Fig 33 In this experiment the ratio of activities (œstriol/16 *epi*œstriol) was approximately 20 1 Statistical calculations showed that there was no significant difference

œstrone and œstriol and, secondly that the remaining 75 per cent is eliminated from the body by other routes or in other forms

Table XIV shows that after the injection of œstriol the pattern of excretion is entirely different Approximately 80 per cent of this steroid is eliminated in the urine unchanged

TABLE XIV

SOME OBSERVATIONS ON THE METABOLISM OF INJECTED ŒSTROGENS

(*After Brown* 1957)

| Œstrogen injected | Total Additional Œstrogen (œstradiol 17β + œstrone + œstriol) as Percentage of Dose injected | Amounts of Individual Œstrogens excreted as Percentage of Total Œstrogen excreted | | |
|---|---|---|---|---|
| | | Œstradiol 17β | Œstrone | Œstriol |
| Œstradiol 17β | 23 | 16 | 40 | 44 |
| Œstrone | 22 | 11 | 43 | 46 |
| Œstriol | 79 | | | 100 |

and there is no additional urinary excretion of œstradiol 17$\beta$ or of œstrone

The œstrogen excretion during the normal menstrual cycle will be discussed later in this chapter (p 173), but at this point it is useful to note that in normally menstruating women the relative proportions of œstradiol 17$\beta$ œstrone and œstriol in urine are very similar to those found in the urine of men and of post menopausal women after the administration of either œstradiol 17$\beta$ or of œstrone Accordingly it may be justifiable to conclude that œstradiol 17$\beta$ and/or œstrone are the principal steroids secreted by the ovary during the cycle and that the total œstrogen content excreted in the urine represents only about one quarter of that secreted in the body

In late pregnancy œstriol constitutes approximately 90 per cent of the total œstrogen excreted while œstradiol 17$\beta$ and œstrone account for only 3 and 7 per cent respectively of the total amount present in the urine These figures are in marked contrast to those obtained in normally menstruating women

production of endogenous œstrogens in the body Accordingly it is necessary to consider the quantitative relationship between the secretion of endogenous œstrogens on the one hand and the excretion of urinary œstrogens on the other

At present it is not possible to measure the actual quantities of the various œstrogens produced by the body and therefore any conclusions drawn must be based on indirect rather than direct evidence Such indirect evidence can be obtained by studies in which pure œstrogens are administered parenterally to suitable human subjects and the amounts of additional œstrogens excreted in the urine are subsequently determined

Numerous investigators in the past conducted such metabolic experiments involving the injection of œstradiol 17$\beta$ and œstrone However the methods used by these workers for the determination of the œstrogens in urine were generally unsatisfactory from the quantitative point of view, and it is therefore doubtful whether much reliance could be placed on the results obtained Recently Brown (1957), using his own method was able to estimate with a much greater degree of accuracy, the œstrogen output after the administration of œstradiol 17$\beta$, œstrone and œstriol Accordingly, Brown's results will form the main basis of the ensuing discussion

The experiments were conducted on two normal male subjects, on two young women with amenorrhœa and on two post menopausal women Each of these individuals was injected intramuscularly with standard doses of pure œstradiol 17$\beta$, œstrone and œstriol After each injection the total additional œstrogen (œstradiol 17$\beta$, œstrone and œstriol) excreted was estimated in each case and a calculation was made of the percentage of the three individual œstrogens as a percentage of the total additional œstrogen The average figures obtained in the six cases are shown in Table XIV

It will be noted that after the injection of either œstradiol 17$\beta$ or of œstrone, approximately one quarter of the administered dose is excreted in the urine as œstradiol 17$\beta$ œstrone and œstriol, and that, in both cases roughly equivalent proportions of œstrone and œstriol are excreted along with relatively smaller amounts of œstradiol 17$\beta$ Accordingly, it seems reasonable to conclude, firstly, that only about 25 per cent of the endogenously produced œstradiol 17$\beta$ and/or œstrone is excreted in the urine as œstradiol 17$\beta$,

time to study on a reasonably quantitative basis the œstrogen excretion in normal and pathological conditions in man Clinical studies with the new methods are at present in progress in many centres and the results obtained will be awaited with considerable interest

**1 Children**

Most investigators agree that the urinary excretion of œstrogens in children and adolescents is relatively low when compared with that of women during reproductive life The most detailed study is probably that of Nathanson *et al* (1941), who used the vaginal smear test in oophorectomised mice and expressed their results in terms of the international standard for œstrone These workers found that, between the ages of three and seven, small quantities of œstrogens were excreted in the urine of both boys and girls and that the amounts present in the two sexes were approximately equal In boys after the age of seven œstrogens continued to be excreted in small quantities and showed little or no rise with the onset of puberty In girls the urinary output of œstrogens rose sharply between the ages of eight and eleven and after eleven it usually became cyclic in character Cyclic œstrogen excretion could precede the menarche by one or two years or could coincide with the first visible signs of secondary sexual development Dorfman *et al* (1937) have emphasised that the œstrogen excretion is related to physical maturity rather than to chronological age

Tillinger *et al* (1957) using Brown s method have recently studied the œstrogen excretion in newborn boys They were unable to detect the presence of œstradiol 17$\beta$ or of œstrone but found measurable quantities of œstriol Investigations of this type in which reliable assay methods are used should in the future yield important information regarding the œstrogen excretion in normal and pathological conditions in children

**2 Normally Menstruating Women**

Numerous investigators using bio-assay methods have measured the excretion of œstrogens during the different phases of the normal menstrual cycle (Smith *et al* 1938 Gustavson *et al* 1938, Pedersen Bjergaard and Tønnesen 1948) Usually two peaks of excretion have been found one at or about the time of ovulation and the other during the luteal phase Curves

and in men and amenorrhœic women injected with œstradiol 17β or œstrone It can only be concluded that a high proportion of the œstriol excreted during pregnancy represents œstriol secreted by the placenta It can also be calculated that the total œstrogen secreted must be greater than the amount excreted in the urine by only about 30 per cent These rough calculations are based on the assumption that the metabolism of secreted œstrogens in the normal non pregnant subject and in the pregnant woman is the same as in the male and in the amenorrhœic woman injected with œstrogens It must be emphasised that this assumption is not necessarily valid

Recently, valuable information regarding the metabolism of administered œstrogens has been obtained by the radio-activity studies of Beer and Gallagher (1955) who administered $^{14}C$ labelled œstradiol 17β to non pregnant human subjects They found that approximately 65 per cent of the administered radio activity appeared in the urine and that the excretion of radio activity in the fæces was negligible This important work makes it possible to draw two main conclusions The first is that the urine is the main route of excretion for œstrogen metabolites, this fact was not appreciated before The second conclusion is that the urine must contain unknown metabolites other than the five known œstrogens and that, from the quantitative point of view, the unknown metabolites are more important than the known metabolites

## THE URINARY EXCRETION OF ŒSTROGENS IN NORMAL SUBJECTS

It has already been stated that until very recently, methods for estimating the urinary œstrogens for clinical purposes were very unsatisfactory Biological procedures based on the Allen Doisy test were generally employed and results were expressed either in animal units' or in terms of the inter national standard for œstrone Such estimations, for reasons already discussed were of only limited significance from the quantitative point of view and little attempt could be made to compare results from different laboratories The recent introduction of reliable chemical techniques for the determina tion of œstrogens in urine has made it possible for the first

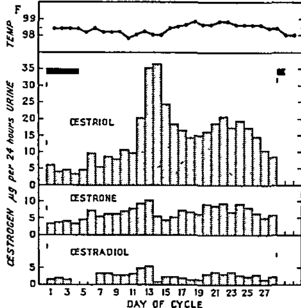



Fig 35
Urinary excretion of œstrogens during the normal menstrual cycle Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1955 *b*)

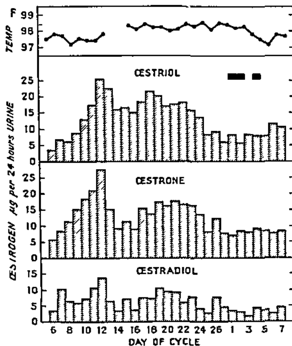



Fig 36
Urinary excretion of œstrogens during the normal

of the same general shape were subsequently obtained by Jayle and Crepy (1952), who used a chemical assay method in which the final determination depended on the Kober reaction Recently Brown (1955 *b*) has studied the œstrogen excretion throughout ten normal cycles Brown's results are probably more meaningful from the quantitative point of view than any previously published, and accordingly his findings will be discussed in some detail Typical curves in individual cases are shown in Figs 35 to 40

It will be noted that in all the subjects studied the amounts of the three œstrogens excreted generally rise and fall together Usually œstradiol 17$\beta$ is excreted in smaller amounts than œstrone but, in a given urine sample, the ratio (œstrone/œstradiol 17$\beta$) is approximately constant at 2 1 On the other hand there appears to be no such constant relationship between the excretion of œstrone and œstriol as shown by the fact that in individual urines the œstriol excretion can be greater than, equal to or less than the corresponding figure for œstrone

During the first seven to ten days of the cycle the excretion of all three œstrogens is low—in most cases 5 $\mu$g or less of each per twenty four hours The levels start to rise on or about the seventh day and reach a well defined maximum at or about the thirteenth day This maximum, which generally coincides with the rise in body temperature has been termed the *ovulation peak* Brown s data therefore suggest that œstrogens are secreted steadily in increasing amounts prior to ovulation and are not suddenly liberated at ovulation as some investigators have previously believed

After ovulation there is a rapid fall in the excretion of all three œstrogens During this phase of the cycle, as well as at ovulation, the changes in the excretion of œstrone and œstradiol 17$\beta$ run parallel but the changes in œstriol excretion tend to lag about twenty four hours behind the other two The significance of this finding will be discussed below The fall in œstrogen excretion is succeeded by a second rise which occurs at or about the twenty first day and is maintained until shortly before the onset of the next menstrual period This second rise can be termed the *luteal maximum* and probably reflects the secretion of œstrogens by the corpus luteum During the luteal maximum the œstriol 'lag phenomena is

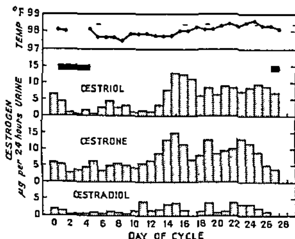



Fig 39 —Urinary excretion of œstrogens during the normal menstrual cycle Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1955 *b*)

Fig 39



Fig 40 —Urinary excretion of œstrogens during the normal menstrual cycle Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1955 *b*)

Fig 40

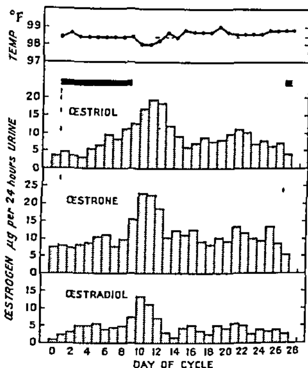



Fig 37

Urinary excretion of œstrogens during the normal menstrual cycle Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1955 *b*)

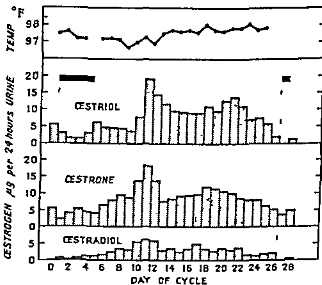



Fig 38

Urinary excretion of œstrogens during the normal menstrual cycle Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1955 *b*)

supports the view that the metabolic formation of œstriol from œstradiol 17$\beta$ or œstrone is a slower process than is the reversible oxidation reduction reaction involving the two latter œstrogens

Brown has shown that at or about the time of ovulation, the rise in the excretion of œstriol lags behind the rise in excretion of œstrone and œstradiol 17$\beta$ by approximately twenty four hours This delay in œstriol excretion, together with the ratios of the three œstrogens found in the urine at this time, is compatible with the view that the maturing follicle secretes œstradiol 17$\beta$ and/or œstrone but not œstriol Evidence regarding the nature of the œstrogens secreted by the ovary during the luteal phase of the cycle is at present conflicting Brown (1955 *b*) originally noted that during the 'luteal maximum the lag in œstriol excretion was not so marked as that found during the follicular phase and suggested that this observation was in keeping with the view that the corpus luteum secreted œstriol as well as œstradiol 17$\beta$ and œstrone However, when the pattern of urinary œstrogen excretion during the normal menstrual cycle was compared with that obtained in normal individuals after the intramuscular injection of standard doses of œstradiol 17$\beta$ œstrone and œstriol no support was found for the contention that the corpus luteum secreted œstriol in addition to the other two œstrogens It has already been mentioned (p 171) that approximately 80 per cent of injected œstriol is recovered in urine as œstriol while only about 10 per cent of injected œstradiol 17$\beta$ or œstrone is so excreted Accordingly it appears reasonable to assume that any œstriol secreted by the ovary would have to be some eight times more active than either œstradiol 17$\beta$ or œstrone in raising the output of urinary œstriol If quantities of œstriol sufficient to obscure the lag phenomenon were indeed secreted by the corpus luteum it would be expected that during the luteal phase of the cycle the excretion of œstriol would be relatively much higher than during the follicular phase Such a marked difference was not found in the ten cycles studied by Brown The mean urinary ratio (œstriol/œstradiol 17$\beta$ + œstrone) was 0 94 1 in the follicular phase and 1 05 1 in the luteal phase These figures are very similar to those obtained following the injection of œstradiol 17$\beta$ or œstrone and indicate that œstradiol 17$\beta$

not apparent Immediately prior to menstruation the excretion of all three œstrogens falls, although the lowest levels are frequently reached several days after the onset of bleeding

Brown (1955 *b*) has found that the pattern of œstrogen excretion is relatively constant from one individual to another but that the actual quantities found in the urine of different subjects vary greatly It can be stated with assurance that single random estimates of œstrogen excretion in menstruating women are of little significance unless the sample has been collected at an accurately known time of the cycle Serial

TABLE XV

ŒSTROGEN LEVELS FOUND AT VARIOUS TIMES DURING THE NORMAL MENSTRUAL CYCLE

*(From Brown 1955 b)*

| Time in Cycle | Œstrogens excreted (µg per twenty four hours) | | | | | |
|---|---|---|---|---|---|---|
| | Average | | | Range | | |
| | Œstriol | Œstrone | Œstradiol 17β | Œstriol | Œstrone | Œstradiol 17β |
| Onset of menstruation | 6 | 3 | 2 | 0-15 | 4 7 | 0-3 |
| Ovulation peak | 27 | 20 | 9 | 13 54 | 11 31 | 4 14 |
| Luteal maximum | 22 | 14 | 7 | 8 72 | 10-23 | 4 10 |

estimations would appear to be necessary in order to arrive at an adequate assessment of ovarian function Average figures obtained at the onset of menstruation, at the ovulation peak and at the luteal maximum are shown in Table XV

NATURE OF OVARIAN ŒSTROGENS —Careful study of Brown's results provides interesting information regarding the nature of the œstrogens secreted by the maturing Graafian follicle and by the corpus luteum

If either œstradiol 17β or œstrone is administered to a man or to a post menopausal or amenorrhœic woman, the subsequent increase which occurs in the urinary excretion of œstriol lags behind the increases in output of œstradiol 17β and œstrone by approximately twenty four hours This finding

figure for total œstrogen excretion was 6 4 $\mu$g per twenty four hours and the levels ranged from 3 2 to 11 2 $\mu$g per twenty four hours Estimations were also made of the excretion of the individual œstrogens and the results were as follows

(*a*) Œstriol Mean excretion 3 3 $\mu$g per twenty four hours, range, 0 6 to 8 6 $\mu$g per twenty four hours
(*b*) Œstrone Mean excretion 2 5 $\mu$g per twenty four hours range 0 8 to 7 1 $\mu$g per twenty four hours
(*c*) Œstradiol 17$\beta$ Mean excretion 0 6 $\mu$g per twenty four hours, range, zero to 3 9 $\mu$g per twenty four hours

### 5 Normal Pregnancy, Labour, Puerperium and Lactation

The œstrogen excretion during normal pregnancy has been studied by many workers including Cohen *et al* (1935), Stimmel (1946), Venning (1948) Bradshaw and Jessop (1953) and Brown (1956) The quantities of œstrogens present in pregnancy urine are relatively large and it has been possible for some time to use chemical rather than biological methods for their determination Œstrogens show an upward trend of excretion during pregnancy and the output reaches very high levels at the end of the gestation period When individual cases are studied most investigators have found a wide fluctuation in levels from day to day and have also reported a considerable variation in excretion from one patient to another at comparable stages of pregnancy After delivery, a rapid fall in output occurs but neither Bradshaw and Jessop (1953) nor Brown (1956) were able to demonstrate any correlation between the œstrogen excretion on the one hand and the onset of labour on the other

At full term the average amounts of the different œstrogens excreted per twenty four hours are approximately as follows

| | |
|---|---|
| Œstradiol 17$\beta$ | 0 75 mg |
| Œstrone | 2 0 mg |
| 16 *epi*œstriol | 0 75 mg |
| 16$\alpha$ hydroxyœstrone | 2 0 mg |
| Œstriol | 30 0 mg |

It will be seen that from the quantitative point of view œstriol is by far the most important of the œstrogens in pregnancy urine It will also be noted that the newly discovered

and/or œstrone are probably the principal ovarian œstrogens secreted during the luteal, as well as the follicular, phase of the normal menstrual cycle

### 3 Normal Men

Small but readily detectable quantities of œstrogens have been demonstrated in male urine by bio assay techniques The main source of production is probably the adrenal cortex, although the testis may also contribute Brown (1955 *c*) has recently studied the œstrogen excretion in twenty nine normal male subjects in the age range twenty to fifty The mean *total œstrogen* excretion was 10 3 $\mu$g per twenty four hours and the figures varied from 6 to 17 8 $\mu$g per twenty four hours Estimations were also made of the excretion of the individual œstrogens and the results were as follows

(*a*) Œstriol Mean excretion, 3 5 $\mu$g per twenty four hours, range, 0 8 to 11 $\mu$g per twenty four hours
(*b*) Œstrone Mean excretion, 5 4 $\mu$g per twenty four hours, range, 3 to 8 2 $\mu$g per twenty four hours
(*c*) Œstradiol 17$\beta$ Mean excretion, 1 5 $\mu$g per twenty four hours, range, zero to 6 3 $\mu$g per twenty four hours

### 4 Menopausal and Post menopausal Women

Œstrogenic activity has been demonstrated in the blood and the urine of menopausal and post menopausal women by numerous investigators using one or other of the modifications of the Allen Doisy test The quantities present are much smaller than those found in women during reproductive life Fluhmann and Murphy (1939) and others have shown that after the menopause the œstrogen excretion may occasionally be cyclic in character

It is reasonable to assume that the main source of œstrogen production in menopausal and post menopausal women is the adrenal cortex Attempts have been made to relate the fall in œstrogen excretion to the development of menopausal symptoms such as flushings but it is by no means definite that such a relationship does indeed exist Œstrogen excretion in the menopausal syndrome is further discussed on page 191

Recently Brown (1955 *c*) has estimated the urinary excretion of œstrogens in twenty post menopausal subjects The mean

about one third of that for œstrone The increase in the urinary levels of œstriol was approximately one thousandfold during the same period

One subject in Brown's series (subject 8) was particularly interesting as it was possible to study her œstrogen excretion over a period of time which included (*a*) a complete menstrual cycle, (*b*) a cycle in which pregnancy occurred (*c*) pregnancy

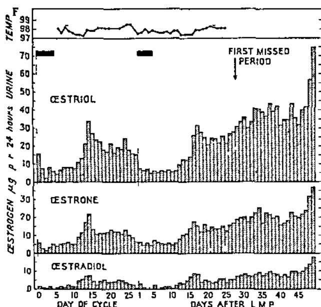



Fig 42

Urinary excretion of œstrogens during a complete menstrual cycle and during a cycle in which pregnancy occurred Basal temperature readings are also shown
■ = menstrual period (*From Brown* 1956)

itself (*d*) labour and the puerperium (*e*) the lactation period and (*f*) the period culminating in the re establishment of normal menstruation In Fig 41 the values for this subject are represented as continuous lines

Fig 42 shows the complete menstrual cycle and the cycle in which pregnancy occurred

It will be noted that the urinary œstrogen levels found in the two cycles were very similar up to and including the ovulation peaks The subsequent luteal rises were also similar until about the twenty fourth day of the cycle After this

œstrogen 16a hydroxyœstrone is a compound of considerable quantitative significance

Recently Brown (1956) has made a careful study of the excretion of œstradiol 17β, œstrone and œstriol in four normally pregnant women His results, which are probably more

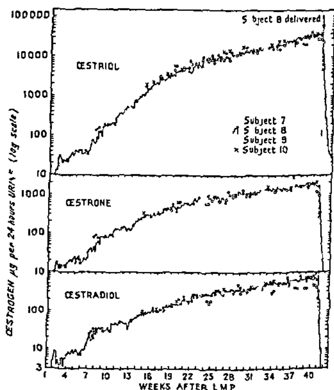



Fig 41

Urinary excretion of œstrogens during normal pregnancy in four subjects (*From Brown* 1956)

meaningful from the quantitative point of view than any previously obtained, are shown in Fig 41

This figure shows that there was a rapid rise in the urinary output of the three œstrogens throughout pregnancy and that the rate of increase was most marked between the sixth and the twentieth weeks The increases in the levels of œstrone and of œstradiol 17β from the luteal maximum to the end of the gestation period were approximately one hundredfold, these rises paralleled each other the excretion of œstradiol 17β being

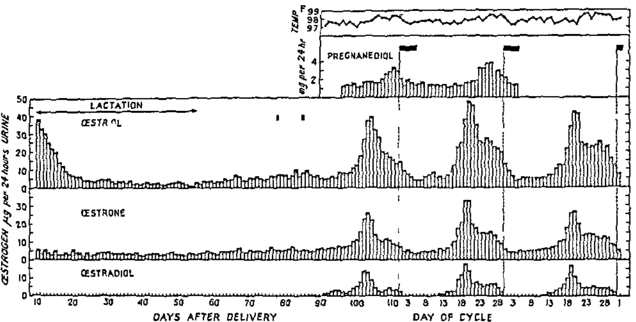



FIG 43

Urinary excretion of œstrogens during the puerperium lactation and three subsequent menstrual cycles Basal temperature readings are also shown ■ = menstrual period (*From Brown* 1956)

time the œstrogen levels continued to increase rapidly in the cycle in which conception had occurred, whereas in the previous cycle the usual luteal maximum was reached and the excretion fell prior to the onset of menstruation

In Fig 43 are presented the results obtained immediately after delivery, during lactation and in the period culminating in the recommencement of normal menstruation

After labour there was a rapid fall in the output of all three œstrogens With œstrone and œstradiol $17\beta$, levels normally encountered in non pregnant subjects were reached within five days In the case of œstriol the rate of elimination was somewhat slower and normal non pregnant levels were not found until some twenty days after delivery

During lactation the œstrogen output was very low and reached a minimum at about fifty days after delivery, when lactation became inadequate and was discontinued Subsequently the quantities of all three œstrogens excreted began to increase slowly until eventually the pattern of excretion characteristic of the menstrual cycle was observed Fig 43 shows the excretion pattern in three cycles

In the first cycle there was evidence, both from basal temperature readings and from the œstrogen and pregnanediol excretion, that ovulation had occurred There was, however, no definite luteal maximum and menstrual bleeding commenced eight days after the ovulation peak In the second cycle the ovulation peak was followed by a somewhat shortened luteal phase of approximately ten days During the third menstrual cycle a normal luteal maximum was found and the œstrogen levels throughout were practically identical with those observed during the cycle studied before conception had occurred

FREE AND COMBINED ŒSTROGENS IN PREGNANCY URINE —*In 1935 Cohen, Marrian and Watson reported* that, whereas practically all of the œstrogen in pregnancy urine is present in the conjugated form throughout the greater part of the gestation period labour or false labour is accompanied by the excretion of 'free or unconjugated œstrogen These workers drew the conclusion that the liberation of free œstrogen from the relatively inactive conjugates might be a factor in the initiation of labour Not all subsequent investigators, however, were able to detect free œstrogens in the

the excretions of both œstrogens of pituitary gonadotrophins are below the normal range while in 'ovarian amenorrhœa' an abnormally low œstrogen output is usually associated with a greatly increased excretion of pituitary gonadotrophins It is customary to divide amenorrhœa into two main types—*primary* and *secondary* In true *primary amenorrhœa* menstruation has failed to occur in patients well beyond the age of puberty The term *secondary amenorrhœa* is applied to the condition in which menstrual function has ceased after having been completely established Both primary and secondary amenorrhœa can be associated with either pituitary or ovarian failure

Hormone levels in cases of amenorrhœa have been estimated by various investigators including Beclere and Simmonet (1949) Kaser (1949) and Pedersen Bjergaard and Tønnesen (1951) The most extensive study yet reported is that of Pedersen Bjergaard and Tønnesen (1951) who determined the urinary excretion of œstrogens and of pituitary gonadotrophins in 110 patients with primary amenorrhœa and 221 patients with secondary amenorrhœa The method of œstrogen assay depended on vaginal cornification in oophorectomised mice and the results were expressed in mouse units the precision of the assays was certainly not high The figures were compared with those obtained by the same assay method in a series of normally menstruating women

In the patients with *primary amenorrhœa* the œstrogen excretion was normal in approximately 20 per cent and abnormally low in approximately 80 per cent Increased excretion levels were not found In 35 per cent of the cases the low œstrogen output was associated with a normal or high excretion of pituitary gonadotrophins and in these patients it is probable that the amenorrhœa was of primary ovarian origin and was not due to pituitary hypofunction In 46 per cent the excretion of both œstrogens and pituitary gonadotrophins was abnormally low and presumably in these subjects the amenorrhœa was of pituitary rather than ovarian origin In the remainder (19 per cent) the hormone levels were within the normal range

In cases of *secondary amenorrhœa* the œstrogen excretion was within normal limits in approximately 33 per cent and abnormally low in approximately 66 per cent In a few patients in this group serial œstrogen assays were conducted,

urine of all patients at the time of delivery and this finding threw doubt on the assumption that 'free' œstrogen had an important physiological function during parturition. In addition, this observation raised the possibility that the 'free' œstrogen determined by Cohen *et al* (1935) might have been an artifact produced either by the hydrolysis of conjugated œstrogen in urine subsequent to voiding or by contamination of urine by other fluids containing unconjugated œstrogen.

This problem was reinvestigated by Clayton and Marrian (1950), who found that 'free' œstrogen was not excreted by women during labour if the urine collections were made by catheter thus avoiding contamination with amniotic fluid or blood clots. They also showed that the enzyme β glucuronidase was present in large quantities in these contaminants. In view of these findings Clayton and Marrian (1950) suggested that the unconjugated œstrogen found by Cohen *et al* (1935) in the urine of women during labour was an artifact produced by the action of the β glucuronidase in amniotic fluid and blood clots on the œstriol glucuronide in urine, and that accordingly little reliance could be placed on estimations of 'free' urinary œstrogens during labour unless special precautions were taken during urine collections.

## THE URINARY EXCRETION OF ŒSTROGENS IN PATHOLOGICAL CONDITIONS

### 1 Disorders of Menstruation

Œstrogen estimations have until now proved of little clinical value to the gynæcologist in the elucidation of problems associated with abnormal menstruation. It is however, probable that with the introduction of better assay methods such determinations will assume greater significance both from the diagnostic and prognostic points of view. Much valuable information should soon be accumulated on the pattern of œstrogen excretion in menstrual disorders and such information may well provide a more rational basis for hormonal therapy in these cases.

(*a*) Amenorrhœa.—In patients with this condition hormone assays are of value in differentiating amenorrhœa resulting from pituitary failure from amenorrhœa due to ovarian deficiency (see also Chap II). In pituitary amenorrhœa

using bio-assay methods found that the œstrogen output was abnormally high in all of fifteen cases studied and that during periods of bleeding the titre did not rise appreciably Other workers including Mayer (1950), have reported normal levels at most stages of the disease In a series of 222 patients with various types of menstrual anomaly other than amenorrhœa Pedersen Bjergaard and Tønnesen (1951) reported that the œstrogen excretion was never increased, that it was reduced in approximately 20 per cent of the cases and was normal in approximately 80 per cent They concluded that abnormalities in œstrogen excretion were much less frequent in this group than in patients with amenorrhœa

The condition of *anovular menstruation* may conveniently be considered at this stage The term refers to cyclic uterine bleeding which occurs in the absence of ovulation and corpus luteum formation There is still much controversy regarding the incidence of anovulatory cycles and whether or not anovular bleeding can be considered as true menstruation The condition is more frequent at the beginning and towards the end of reproductive life but may occur at any time during the child bearing period, indeed it is probable that anovular cycles are much more frequent than was at one time supposed In anovular menstruation bleeding occurs from a proliferative and not from a secretory endometrium The most reliable method of arriving at a diagnosis is by endometrial biopsy although the finding of a monophasic temperature chart is suggestive of the condition

Brown (1957) has recently studied the urinary excretion of œstradiol 17β œstrone and œstriol in a small number of patients with anovular menstruation In Fig 44 are shown the results obtained in a patient aged nineteen who presented with a history of irregular menstrual bleeding and heavy menstrual loss Assays were conducted over a period of time which included the phases of menstrual bleeding

It will be seen that the pattern of œstrogen excretion was different to that observed during the normal menstrual cycle There was no rhythmic fluctuation in œstrogen output and no ovulation peak could be demonstrated There was no apparent correlation between the œstrogen excretion on the one hand and the episodes of bleeding on the other Endometrial biopsies taken at intervals during the study gave an

considerable variations in excretion, sometimes of a cyclic nature, were observed

(*b*) DYSFUNCTIONAL UTERINE HÆMORRHAGE —This is a loose term which is used to describe all forms of abnormal bleeding for which no gross organic cause can be found Various classifications for dysfunctional uterine hæmorrhage have been proposed but none are completely satisfactory The subdivision can be based on symptomatology on the histological appearance of the endometrium or on the presence or absence of ovulation In the present discussion consideration will be given mainly to the anovular types of dysfunctional uterine hæmorrhage

The condition known as *metropathia hæmorrhagica* is a member of this group which is encountered relatively often in clinical practice The characteristic symptom of this disease is continuous bleeding which is often profuse and may last for many weeks The hæmorrhage is usually painless and may be preceded by a period of amenorrhœa The condition may occur at any age but is more frequent just before the menopause or immediately after the menarche The characteristic findings in the ovary are the presence of multiple follicles and the absence of any active luteal tissue The endometrium in typical cases shows cystic hyperplasia without any evidence of secretory activity The appearance is one of an exaggerated proliferative phase and it is generally assumed that the endometrial reaction results from prolonged and unopposed stimulation by œstrogenic hormones

Various theories have been advanced to explain the bleeding in cases of metropathia hæmorrhagica The preliminary amenorrhœic phase may result from the maintenance of a blood œstrogen level above the so called bleeding threshold and the subsequent bleeding may occur when the circulating œstrogens fall below this level This view has not so far been confirmed by means of hormone assay It is also possible that the continuing œstrogen secretion by the ovaries builds up the endometrium to a point at which larger and larger amounts of œstrogen are required to sustain it If these are not produced the endometrium outgrows its œstrogen supply and breakdown occurs

Little information is at present available on the œstrogen excretion in patients with metropathia Furuhjelm (1948),

## 2 The Menopausal Syndrome

The symptoms which arise at the menopause are generally believed to result primarily from a decline and eventual failure of ovarian function Various theories have been advanced regarding the relationship of hormones to menopausal symptoms and the evidence for and against these hypotheses has been reviewed by numerous workers including Fluhmann (1944) Heller *et al* (1944) and others One of the theories postulates that the symptoms are caused directly by the over production of pituitary gonadotrophins, this view has already been considered in Chapter II The theory of œstrogen lack which will be briefly mentioned below postulates that the menopausal syndrome results from a decrease in the œstrogen titre of body fluids

The main points in favour of the view that a diminution in circulating œstrogens can cause menopausal symptoms are first that the latter can occur when evidence of ovarian failure is present and secondly that such symptoms are usually rapidly relieved by œstrogen therapy However as emphasised by Fluhmann (1944) there are serious objections to accepting œstrogen withdrawal as the sole cause of menopausal symptoms In the first place when assays of urinary œstrogens have been performed in menopausal patients no correlation has been found between the œstrogen excretion on the one hand and the presence or severity of symptoms on the other Secondly, menopausal symptoms may appear some time before menstruation ceases and in such individuals it is reasonable to suppose that the titre of circulating œstrogens would not be abnormally low Finally in patients with panhypopituitarism in whom the urinary excretion of œstrogens is generally very low menopausal symptoms such as flushings are seldom if ever encountered

It is obvious that considerable controversy still exists regarding the part played by the œstrogens in the ætiology of menopausal symptoms The problem remains one for future elucidation

## 3 Ovarian Tumours

The *œstrogenic tumours of the ovary* are composed of cells of the granulosa or thecal type or of a mixture of these Most but by no means all of these neoplasms exhibit œstrogenic activity The most important members of the group are the

appearance characteristic of the proliferative phase of the cycle, while urinary pregnanediol determinations were relatively low throughout and indicated the absence of a functional corpus luteum

(*c*) DYSMENORRHŒA —It is not known with certainty if œstrogens play any part in the ætiology of this condition Large doses of natural and synthetic œstrogens, administered during the first half of the cycle, prevent ovulation and the succeeding period, being anovular, is painless Hormone assays by reliable methods in patients with dysmenorrhœa will be awaited with interest

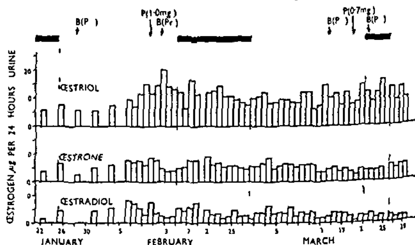



FIG 44

Urinary excretion of œstrogens in a patient with anovular menstruation ■ = episodes of bleeding B(Pr) = Biopsy characteristic of proliferative phase P = pregnanediol ' indicates the time of the biopsies and of the pregnanediol determinations (*From Brown* 1957)

(*d*) PRE MENSTRUAL TENSION —This condition is generally regarded as an exaggeration of the normal menstrual molimina The patient is *conscious of nervous tension* and may show depression, insomnia and marked emotional instability Symptoms usually develop during the seven to ten days prior to menstruation Little is known of the ætiology of the condition, but the suggestion has been made that the clinical features result from the presence in the blood of abnormally large quantities of œstrogens At the time of writing no reliable information is available on the œstrogen excretion in patients with this syndrome

of the lesion in the contralateral ovary was indicated by a rise in output In cases of isosexual precocity in girls œstrogen assays may be of value in differentiating the rare granulosa cell tumour from the much more frequent constitutional precocious puberty In the latter condition the œstrogen excretion is generally within normal limits The subject of œstrogen excretion in patients with granulosa cell tumour has recently been reviewed by Bishop (1954)

In the majority of cases of *thecoma* reported in the literature, œstrogen assays have not been performed No data is at present available regarding the œstrogen excretion in patients with *fibroma* or with the *Brenner tumour*

*Chorionepithelioma* of the ovary is exceedingly rare and is not generally included in the group of functioning ovarian tumours A case of this tumour was reported by Seckel (1946) and in this patient an abnormally high excretion of urinary œstrogens was found

## 4 Fibromyoma of the Uterus

Lipschutz (1950) has shown that long continued administration of œstrogens to guinea pigs causes the development of fibromyomatous growths of the uterus and other abdominal organs It is however doubtful if œstrogenic stimulation is a factor in the production of uterine fibroids in women and the ætiology of the condition remains obscure

At the time of writing hormone assays by reliable methods have not been conducted in patients with fibromyomata Such studies will be awaited with interest

## 5 Genital Carcinoma in Women

There has been much controversy as to whether the œstrogenic hormones are responsible for the development of uterine cancer in man and animals Evidence from animal experiments is conflicting and varies greatly with the species studied In monkeys continuous œstrogen administration for many months may cause squamous metaplasia of the cervical epithelium but the lesions seldom if ever become malignant (Hisaw and Lendrum 1936) In rabbits carcinoma of the uterus has occasionally been reported following prolonged œstrogen therapy Gardner and his colleagues (Gardner 1937, Gardner *et al*, 1953) have shown that in mice, marked

*granulosa cell tumour* and the *thecoma*, but probably the *fibroma* and the *Brenner tumour* should also be included in this category. The whole subject of functioning ovarian tumours has recently been reviewed by Matthew *et al* (1956) on whose article the following discussion is mainly based.

The *clinical features* of œstrogenic tumours are very variable and depend on three main factors. These are

(*a*) The presence of an ovarian tumour. The symptomatology is influenced by such variables as the size of the tumour, its degree of malignancy and the presence or absence of such local complications as torsion of the pedicle, hæmorrhage and rupture.

(*b*) The hormonal activity of the tumour. This subject will be discussed further below.

(*c*) The presence of associated pathological lesions which may or may not be directly attributable to the functioning nature of the tumour. In this group are included such conditions as endometriosis, uterine fibroids, carcinoma of the breast and carcinoma of the endometrium. It has been suggested that these lesions might arise as a result of prolonged and excessive secretion of œstrogens by the ovarian neoplasm.

THE HORMONAL ACTIVITY OF ŒSTROGENIC TUMOURS.—At present little is known regarding the site of origin of tumour œstrogens, but it is probable that both the granulosa cells and the thecal cells are capable of elaborating these substances. One of the principal sites of action of œstrogenic hormones is the uterine endometrium, and in most but not all patients with functioning ovarian tumours endometrial proliferation occurs. It is reasonable to suppose that, in a given case, the endometrial reaction is governed by such factors as the nature and physiological activity of the tumour œstrogens, the duration of œstrogenic action and the inherent reactivity and sensitivity of the endometrium itself to hormonal stimulation.

At the time of writing little or no reliable information is available in the literature on the excretion of œstrogens in patients with *granulosa cell tumour*. Studies in individual cases have been reported by various workers including Glass and McKennon (1937) and Stohr (1942), but in all instances biological methods of assay were employed. In many patients but not in all, abnormally high œstrogen concentrations were found in both blood and urine. Removal of the tumour resulted in a sharp fall in œstrogen excretion, while recurrence

after prolonged treatment with œstrogens (Geschickter, 1945) On the other hand guinea pigs and monkeys seldom, if ever, show mammary carcinoma as a result of treatment with exogenous œstrogens

There has been much speculation on the possible relationship of endogenous œstrogen secretion to mammary carcinoma in women Breast carcinoma is much more frequent in patients who experience a late menopause than in those in whom the menopause occurs between the ages of forty and fifty Carcinoma of the breast occurring during pregnancy tends to be rapidly progressive, and this has been attributed to the high levels of circulating œstrogens which are found in pregnant subjects Some patients with mammary carcinoma improve markedly following oophorectomy or ovarian irradiation and it is widely believed that this improvement results from a reduction in the amount of œstrogens present in body fluids On the other hand there is very little evidence that exogenously administered œstrogens are carcinogenic in human subjects Great numbers of patients are treated with œstrogens each year but the number of cases of mammary carcinoma reported in the literature following prolonged œstrogen therapy is very small indeed In view of this finding it appears unlikely that the administration of exogenous œstrogens *per se* is an important ætiological factor in cancer of the human breast

The assay of urinary œstrogens in cases of mammary carcinoma might prove of value for a number of reasons In the first place it would be of importance to determine the pattern of endogenous œstrogen excretion in these patients and to ascertain whether or not this differs in any way from that of control subjects in a comparable age group

Secondly œstrogen assays might yield important information with regard to the form of therapy which should be employed in a given case For example it would seem unlikely that patients in whom the endogenous œstrogen levels were abnormally high would benefit from treatment with exogenous œstrogens but on the other hand such cases might well be improved by procedures such as oophorectomy ovarian irradiation or adrenalectomy which are designed to reduce the amounts of physiologically active œstrogens in the body Finally it would be of considerable interest to study the metabolism of exogenously administered œstrogens in patients

proliferation of the vaginal and uterine epithelium may follow the administration of œstrogens, in some strains of mice carcinoma of the cervix may develop when the animals are so treated for one year or more

Much has been written on the possible relationship of œstrogens to genital cancer in women With regard to *carcinoma of the body of the uterus* it has been shown that this condition is more frequent in patients who experience a late menopause and who are presumably exposed to œstrogenic stimulation for an abnormally long period of their life Furthermore, some authors have reported that the incidence of endometrial carcinoma is unusually high in patients with œstrogenic tumours of the ovary and have concluded from this observation that the tumour œstrogens are actively carcinogenic Other workers, however, hold the view that the evidence at present does not warrant such a definite conclusion It is a matter of common clinical experience that the incidence of *carcinoma of the cervix* is considerably higher in multiparous women than in nullipara or in primipara The suggestion has been made that the multiparous patient is more susceptible to this form of cancer as a result of exposure during each of her pregnancies to abnormally high concentrations of circulating œstrogens

Until now œstrogen assays by reliable methods have not been conducted in cases of genital carcinoma Such studies are urgently required, and it is to be hoped that they will yield information which will be of importance in relation to the ætiology, prognosis and treatment of patients with this disease

**6 Mammary Carcinoma**

Œstrogens are potent stimulators of cellular growth and division in the breast In certain strains of female mice prolonged administration of œstrogens causes hyperplasia and metaplasia of the mammary cells, culminating eventually in mammary carcinoma, in male mice of the same susceptible strain œstrogens will also induce cancer of the breast (Lacassagne 1932 Burns and Schenken, 1940, Shimkin and Wyman, 1946) It is generally believed that, in the ætiology of breast cancer in mice, both œstrogenic stimulation and heredity play a part Rats are much less susceptible to spontaneous mammary cancer but can develop the condition

it is possible that such estimations might yield interesting information regarding the ætiology of the disease

## 8 'Senile' Vaginitis

This is a low grade chronic pyogenic infection occurring in post menopausal subjects The condition generally improves rapidly on œstrogen therapy and this has prompted the suggestion that œstrogen lack is a factor in its pathogenesis The validity of this theory should be tested by means of hormone assay

## 9 Abnormal Pregnancy

(*a*) Pre eclamptic Toxæmia —In the majority of cases the excretion of œstrogens is below the normal range (Smith and Smith 1934 1948, Browne *et al* 1938) Normal values and high values are encountered infrequently Smith and Smith (1948) have emphasised that the curve of excretion in individual patients is more informative than the actual level at any one time during pregnancy There appears to be no definite correlation between the low œstrogen level on the one hand and any specific clinical feature such as œdema hypertension or albuminuria on the other Rakoff (1939) has reported that the œstrogen levels in blood and urine tend to rise as the clinical condition of the patient improves The decreased excretion of œstrogens in pre eclamptic toxæmia may result from a number of causes of which diminished production of the hormone by the placenta would appear to be the most important

It will be remembered that a low serum œstrogen was one of the abnormalities noted by Smith and Smith in cases of pre eclamptic toxæmia This finding was one of the factors which prompted a therapeutic trial with œstrogens and progesterone in patients with this disease The Smith theories in relation to the ætiology and treatment of pre eclamptic toxæmia are not now generally accepted and will not be further discussed

(*b*) Diabetic Pregnancy —Few œstrogen assays have so far been performed in pregnant diabetics In cases uncomplicated by pre eclamptic toxæmia the excretion has usually been within the normal range (Rubin *et al*, 1946), but in patients showing both diabetes and toxæmia abnormally low titres for the stage of pregnancy have been reported

with mammary carcinoma and to determine whether this differs in any way from that in normal individuals

At the time of writing, a number of centres in the United Kingdom and elsewhere are conducting urinary œstrogen assays in cases of breast cancer, using the newer and more reliable chemical methods of determination which have recently been developed At this juncture it would be premature to make any definite statement with respect to the diagnostic and prognostic value of such assays, although some of the preliminary results obtained may be briefly considered

Strong *et al* (1956) studied the excretion of œstradiol 17β, œstrone and œstriol in thirteen patients who had previously undergone bilateral adrenalectomy and bilateral oophorectomy for mammary carcinoma The patients were classified into two clinical groups, depending on their response to operation It was found that the pattern of urinary œstrogen excretion in patients who responded favourably to the operations did not differ from that in patients who showed no improvement after removal of both adrenals and ovaries Bulbrook and Greenwood (1957 *a* *b*) using Brown s method, investigated the effect of bilateral oophorectomy, bilateral adrenalectomy and hypophysectomy on the urinary excretion of œstrogens in patients with mammary cancer They showed that œstrogen excretion usually continued after oophorectomy and that, in a proportion of cases, it was not completely abolished by a subsequent adrenalectomy Persistence of œstrogen production, as judged by the findings on urinary assay, was also demonstrated in post menopausal and oophorectomised patients subjected to hypophysectomy

## 7 Endometriosis

This condition may be defined as the presence of endometrial elements in an abnormal situation The symptomatology is largely due to the action of ovarian hormones upon the ectopic endometrium The suggestion has been made that the cell metaplasia characteristic of endometriosis is initiated by œstrogenic stimulation during the active child bearing period of a woman s life Some support for this view comes from the observation that the condition is not uncommon in patients with functioning ovarian tumours Œstrogen assays by reliable methods have not yet been reported in cases of endometriosis

was one of feminisation Œstrogen determinations have been undertaken in a small number of patients with this type of feminising tumour (Simpson and Joll 1938, Luft and Sjogren 1949, Diczfalusy and Luft, 1952 Dohan *et al*, 1953) In all the cases reported, the urinary excretion was found to be abnormally high

Probably the most detailed study so far reported on the œstrogen excretion in a case of feminising adrenocortical tumour is that of Diczfalusy and Luft (1952) These workers purified their extracts by countercurrent distribution and subsequently estimated the individual œstrogens by fluorimetry The urine extracts were shown to contain œstradiol 17β œstrone and œstriol in abnormally large amounts

Migeon and Gardner (1952) have estimated the excretion of urinary œstrogens in cases both of adrenocortical tumour and of adrenocortical hyperplasia Two methods of assay were used One was a chemical procedure, slightly modified from that of Jailer (1948) in which the final determination was made by fluorimetry, the other was a bio assay method depending on the enlargement of the uterus in intact immature mice It was claimed that urinary œstrogen determinations conducted before and after cortisone therapy might be of value in differentiating between the two diseases This claim was based on the observation that in patients with adreno cortical hyperplasia cortisone reduced the output of urinary œstrogens to normal levels whereas in cases of adrenocortical tumour, therapy by cortisone did not appreciably alter the excretion of these hormones

It should be emphasised that the assay methods used by Migeon and Gardner (1952) in their study are not very satis factory from the quantitative point of view Their work should be repeated with the more reliable assay methods for œstrogens now available

## 12 Prostatic Carcinoma

May and Stimmel (1948 1955) have claimed that the metabolism of administered œstrogens differs in patients with prostatic carcinoma from that in normal men These workers administered œstrone parenterally to both types of case and showed that, whereas the major œstrogen excreted in the urine of normal subjects was œstrone itself the principal steroid

(c) HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA.—Information regarding the œstrogen excretion in patients with these diseases is at present very scanty. Studies have been reported by a small number of investigators, including Smith and Werthessen (1941), Payne (1941) and Hinglais and Hinglais (1949). In all cases bio assay methods were used. Levels have been reported as normal, abnormally high or unusually low. It is obvious that further work is necessary before any definite statement can be made on the diagnostic value of œstrogen assays in such patients.

(d) THREATENED AND RECURRENT ABORTION.—Smith and Smith (1935) found normal levels in all cases studied while Browne *et al.* (1939) obtained abnormally low values in patients who subsequently aborted. Kaser and Eichenberger (1949) and Mayer (1950) reported that the œstrogen excretion decreased rapidly in cases of inevitable abortion or fœtal death. A similar conclusion was reached by Jayle and Plantureux (1953).

The problem of œstrogen excretion in threatened and recurrent abortion should be reinvestigated now that more reliable assay methods are available. It is not unlikely that such a study would yield information of clinical importance.

## 10. Testicular Tumours

Information on the pattern of œstrogen excretion in patients with testicular tumours is extremely scanty. Hamburger (1938) has reported that the œstrogen output in cases of *seminoma* is generally within normal limits while patients with *chorionepithelioma* of the testis may excrete abnormally large amounts. The very rare *Leydig cell tumours* are usually masculinising in character but may occasionally be associated with feminising symptoms such as *gynæcomastia*. The œstrogen excretion in such patients has not yet been adequately studied nor have determinations been reported in the even more uncommon *Sertoli cell tumours* of the testis.

## 11. Adrenocortical Tumours and Adrenocortical Hyperplasia

The great majority of adrenocortical tumours produce masculinising effects but approximately twenty cases have been reported in the literature in which the clinical picture

is frequently no higher than that found when similar metabolic experiments are conducted in subjects with normal liver function In the opinion of May and Stimmel (1955) impairment of œstrogen metabolism in cases of portal cirrhosis is indicative of a very poor prognosis as this abnormality is not usually found unless the patient is in an almost moribund condition

## Summary and Conclusions

Œstrogen assays in human urine should now be conducted by chemical rather than by biological methods Chemical methods depending on colorimetry are at present more reliable than fluorimetric techniques Satisfactory methods are not yet available for the quantitative determination of œstrogens in blood

For the estimation of urinary œstrogens in clinical conditions the methods described by Brown (1955 *a*) and by Bauld (1956) are recommended These techniques measure with reasonable accuracy the three œstrogens, œstradiol 17$\beta$ œstrone and œstriol, but do not determine the more newly discovered urinary œstrogens 16 *epi*œstriol and 16$\alpha$ hydroxyœstrone

Urinary œstrogen estimations can provide useful information regarding the secretion of endogenous œstrogens by the body

Reliable figures are now available for the excretion of œstradiol 17$\beta$ œstrone and œstriol during the normal menstrual cycle Readings are low in the early proliferative phase and just prior to the onset of menstruation Peaks of excretion occur at or about the time of ovulation and during the luteal phase In normal pregnancy relatively large quantities of œstrogens are excreted, the levels gradually increase throughout pregnancy and fall rapidly after delivery Readings remain low during the puerperium and in the lactation period The œstrogen excretion in men in post menopausal women and in children is relatively low and is probably mainly of adrenal origin

Urinary assays of œstrogens may sometimes be helpful in the investigation of menstrual abnormalities in the diagnosis of functioning ovarian tumours and in the study of various abnormalities during pregnancy It is possible that in the

found in the urine of patients with prostatic carcinoma was oestriol. The characteristic cancer pattern was not altered by subsequent orchidectomy and was not found in patients with other forms of malignant disease.

The observations of May and Stimmel may well be of considerable interest in relation to the aetiology of prostatic cancer in man. Further studies along similar lines would be desirable.

## 13 Liver Disease

Many observations both in animals and in humans support the view that the liver plays an important role in the metabolism of oestrogens. For example, it is well known that oestrogens when incubated with liver slices *in vitro* rapidly lose much of their biological activity. Furthermore, it is widely believed that, in patients with liver disease, certain clinical features such as gynaecomastia, testicular atrophy, spider naevi and palmar erythema result from the presence in the circulation of abnormally large quantities of oestrogenic hormones.

Little information is available in the literature regarding the urinary excretion of endogenous oestrogens in patients with portal cirrhosis. The most careful investigation is that recently conducted by Cameron (1957). This worker, using Brown's method, estimated the urinary output of oestradiol 17$\beta$, oestrone and oestriol in twelve patients with chronic liver damage. In the majority of cases the readings obtained were within the normal range and in only a few individuals were the levels abnormally high.

A number of studies have been made of the metabolic fate of injected oestrogens in patients with hepatic cirrhosis. The subject has recently been reviewed by May and Stimmel (1955). Earlier work in which biological methods of assay were employed indicated that when oestradiol 17$\beta$ and oestrone were administered to cirrhotics as much as 80 per cent of the injected dose was subsequently recovered in the urine. These results were in keeping with the view that in the presence of liver damage, oestrogen inactivation and degradation were considerably impaired. However, later work with more reliable chemical methods of estimation has not borne out this conclusion, and it has been shown that the total amount of injected oestrogen recovered from the urine of cirrhotic patients

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future, œstrogen estimations, conducted by improved methods, will be of diagnostic value in such conditions as genital carcinoma, mammary carcinoma, prostatic carcinoma and adrenocortical tumours

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CHAPTER X

# *Progesterone and its Metabolites*

## INTRODUCTION

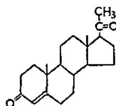
IN 1929 Corner and Allen showed that lipoid extracts prepared from the corpora lutea of swine were capable of causing progestational changes in the uterine endometrium of castrated rabbits This observation indicated strongly that the corpus luteum secreted a specific hormone and in 1934 this hormone which is now known as progesterone, was isolated as a chemically pure substance from the luteal tissue of sow s ovaries The isolation was accomplished almost simultaneously by four different groups of workers (Butenandt *et al*, 1934 Slotta *et al*, 1934, Allen and Wintersteiner 1934, Hartmann and Wettstein, 1934) Very shortly afterwards the structure of the hormone was completely elucidated by Butenandt and Schmidt (1934) and by Fernholz (1934)

In more recent years progesterone has been isolated in relatively small quantities from sources other than the ovary These have included ox adrenal tissue and human placental tissue The presence of the hormone has also been detected in human blood during pregnancy and during the luteal phase of the menstrual cycle Attempts to demonstrate progesterone in human urine have usually been unsuccessful although Ungar *et al* (1951) were able to detect its presence in urine extracts after the administration of very large doses of the hormone to patients with rheumatoid arthritis

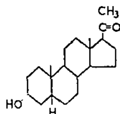
Progesterone and its metabolites are all derivatives of two parent hydrocarbons 5α pregnane (*allo*pregnane) and 5β pregnane the structural formulæ of which are shown in Fig 45

These two substances differ only in the spatial configura tion of the hydrogen atom at C 5 Among the metabolic reduction products of progesterone, those with the 5β configuration are much the more important from the

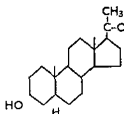
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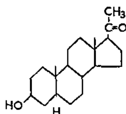

pregn 4 ene 3 20 dione
*(progesterone)*



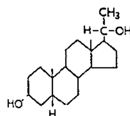

5β-pregnan 3α ol 20-one
*(pregnanolone)*



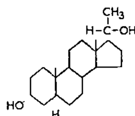

5α pregnan 3α ol 20-one
*(allopregnanolone)*



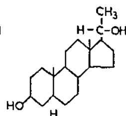

5α pregnan 3β ol 20 one



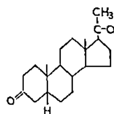

5β pregnane 3α 20α diol
*(pregnanediol)*



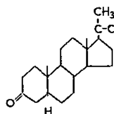

5α pregnane 3α 20α diol
*(allopregnanediol)*




5α pregnane 3β 20α diol




5β pregnane 3 20 dione
*(pregnanedione)*




5α pregnane 3 20 dione
*(allopregnanedione)*

FIG 46

Structural formulæ of progesterone and of some of its urinary metabol tes

quantitative point of view In this group are included the two compounds 5$\beta$ pregnane 3$\alpha$ 20$\alpha$ diol (pregnanediol) and 5$\beta$ pregnan 3$\alpha$ ol 20 one (pregnanolone)

In Fig 46 are shown the structural formulæ for progesterone and for some of its urinary metabolites

Only pregnanediol, *allo*pregnanediol and pregnanolone have so far been definitely proved to be metabolic reduction products of progesterone, but it is very probable that the other compounds listed have a similar origin

Progesterone metabolites can be divided into three groups, depending on their degree of reduction These are (1) the

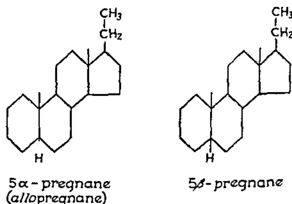



Fig 45
Structural formulæ of 5α pregnane (*allo*pregnane) and 5β pregnane

*pregnanediones*, (2) the *pregnanolones* and (3) the *pregnanediols* These three groups of compounds contain respectively two four and six more hydrogen atoms than does progesterone itself

Very small quantities of *pregnanediones* have been demonstrated in extracts of human pregnancy urine There is no reason to believe that these substances are of any physiological significance, and at present satisfactory methods are not available for their quantitative determination in blood and urine

The most important compound among the *pregnanolones* is 5$\beta$ pregnan 3$\alpha$ ol 20 one (pregnanolone) This substance is excreted in gradually increasing amounts during normal pregnancy and it can be recovered from male urine following

## 2 Placenta

Progesterone has been isolated from human placental tissue by Salhanick *et al* (1952), Pearlman and Cerceo (1952) and Diczfalusy (1952) while Pearlman and Thomas (1953) have demonstrated the presence of the hormone in human placental blood Although large amounts of urinary progesterone metabolites are excreted throughout pregnancy, the progesterone content of the placenta, as determined by existing methods, is relatively low This finding is in keeping with the view that the placenta produces but does not store progesterone During pregnancy the placenta gradually takes over from the ovary the production of progesterone and in the second and third trimesters of pregnancy this organ is probably the dominant source of the hormone

## 3 Adrenal Cortex

In 1938 Beall and Reichstein isolated progesterone from ox adrenal tissue and although the hormone has not yet been identified in human adrenals, there is a reasonable amount of indirect evidence to suggest that this organ in man is capable of progesterone secretion In men in post menopausal women and in women during the follicular phase of the menstrual cycle small but measurable quantities of urinary pregnanediol are excreted (Engel *et al* 1941, Westphal, 1944 Klopper *et al* 1955) It is widely believed that this material which has been termed *adrenal pregnanediol* is derived from some precursor or precursors—possibly progesterone—which have been secreted by the adrenal cortex Recent studies by Klopper *et al* (1957) provide strong support for this view These workers have demonstrated that the intravenous administration of ACTH to men and to post menopausal and oophorectomised women results in a sharp increase in the urinary excretion of pregnanediol and that in patients who have been both oophorectomised and adrenalectomised the urinary pregnanediol output generally falls to levels which are indistinguishable from zero These observations suggest that urinary pregnanediol estimations may be a useful means of assessing adrenocortical function in men and in post menopausal women

the oral administration of progesterone At the time of writing satisfactory methods are not available for the quantitative determination of pregnanolone or of its stereo isomers in the body fluids of man

The group of *pregnanediols* includes the substance 5β pregnane-3α 20α diol (pregnanediol) which from the quantitative point of view is by far the most important metabolic reduction product of progesterone This compound was first isolated from pregnancy urine by Marrian in 1929 and was subsequently shown to have a structure closely resembling that of progesterone itself Unlike the latter, however, pregnanediol is not progestationally active The steroid is excreted in relatively large quantities during pregnancy and in smaller amounts during the luteal phase of the menstrual cycle Small but measurable amounts of pregnanediol are also present in the urine of males, of post menopausal women and of women during the follicular phase of the menstrual cycle As first demonstrated by Venning and Browne (1936) pregnanediol is excreted in urine conjugated with glucuronide acid as *sodium pregnanediol glucuronide* (NaPG) Stereo-isomers of pregnanediol which have been isolated from pregnancy urine include 5α pregnane 3α 20α diol (*allo*pregnanediol) and 5α pregnane 3β 20α diol (Fig 46) These substances occur in relatively small amounts and are probably of little physiological importance Satisfactory methods are not at present available for their quantitative estimation

## SOURCES OF PROGESTERONE IN THE BODY

*In human subjects progesterone is produced by the ovary* the *placenta* and probably by the *adrenal cortex*

### 1 Ovary

It is generally believed that progesterone is secreted by the granulosa lutein cells of the corpus luteum, but it is also possible that the cells of the theca interna are capable of elaborating the hormone Many investigators have shown that pregnanediol is excreted in relatively large quantities during the luteal phase of the menstrual cycle This material, which may be termed *ovarian pregnanediol*, is derived from the progesterone secreted by the luteal tissue of the ovary

There has been much controversy regarding the extent to which other steroids, in particular the œstrogens, interfere with the Hooker Forbes test Salhanick *et al* (1951) found that œstradiol 17$\beta$ in relatively small amounts was capable of inhibiting the action of progesterone on the stromal nuclei of ovariectomised mice This observation led these workers to suggest that in assays conducted on untreated blood samples containing both œstrogens and progesterone falsely low readings for the progesterone concentration might easily be obtained Zarrow and Neher (1953) showed that œstrone as well as œstradiol 17$\beta$ could modify the response of the endometrium to progesterone They stated, however, that the quantity of œstrogenic material present in blood was insufficient to interfere with the response and concluded that the method could probably be employed for progesterone determinations in untreated blood

In view of the low degree of precision and questionable specificity of the Hooker Forbes test, it is not possible to recommend the method at least in its present form, for the quantitative determination of progesterone in blood

(*b*) Methods employing Rabbits —Various assay methods for progesterone employing rabbits have been proposed One of the more popular of these was described by McPhail (1934) and depended on the production of progestational changes in the uterine endometrium This test and others like it are too insensitive for clinical use and will not be further discussed

**2 Chemical Methods**

Numerous attempts have been made to estimate the progesterone content of peripheral blood but in earlier studies such as those by Haskins (1950) and by Butt *et al* (1951) negative results were reported

Recently Zander and Simmer (1954) have developed a micro method which is sufficiently sensitive to estimate the blood concentration of progesterone in human subjects The main steps in this procedure are as follows (*a*) extraction of progesterone with a mixture of ethanol and ether (*b*) distribution of the concentrated extract between water and ethyl acetate in order to remove non lipoid material (*c*) precipitation of lipoid impurities by dissolving the dry residue from the ethyl acetate phase in methanol and by chilling to $-15°$ C,

## METHODS OF ASSAY OF PROGESTERONE IN BLOOD

Until recently an assessment of the endogenous production of progesterone by the ovary, adrenal cortex or placenta has depended almost entirely on the measurement of urinary pregnanediol excretion. Within the past few years, however, methods have been developed for the estimation of progesterone itself in blood. Such techniques may become of importance in clinical work as they provide a more direct means than has previously existed of studying one aspect of ovarian and placental function in health and disease.

Assays of blood progesterone can be performed either by *biological* or by *chemical* methods. For reasons which will be discussed below it is probable that in future investigations chemical techniques will entirely replace bio assay procedures.

### 1 Biological Methods

(*a*) THE HOOKER FORBES TEST (Stromal nuclear hypertrophy in mice).—In 1947 Hooker and Forbes described a method which was claimed to be sufficiently sensitive to detect the presence of progesterone in blood. The technique involves the intra uterine injection of very small amounts of the hormone in ovariectomised mice followed by histological examination of the endometrium. When stimulated by progesterone the stromal nuclei enlarge, their nucleoli become conspicuous and their chromatin particles become evenly distributed; in untreated animals on the other hand the nuclei are shrunken, the nucleoli are small and inconspicuous and the chromatin particles remain clumped. This method probably has a low degree of precision but is claimed to be very sensitive, positive responses being obtained with total doses as small as 0 0002 µg of progesterone per animal. In their earlier publications Hooker and Forbes stated that their method was reasonably specific. However, subsequent investigations showed that this claim was not justified and the original authors now suggest that the technique measures progestogens or 'progestin activity' rather than progesterone itself. In a study of blood levels of 'progestogens' in human pregnancy and in the luteal phase of the menstrual cycle in women and in monkeys Forbes (1951) *obtained values ranging from* 4 to 8 µg per ml

blood Figures were presented for the concentration of blood pregnanediol before and after the intravenous administration of progesterone to healthy male subjects This work was subsequently quoted in review articles by Samuels and West (1952) and by Lieberman and Teich (1953) Somewhat surprisingly, however in view of the great importance of the original claims the details of the actual technique employed have not yet been published

## METHODS OF ASSAY OF PREGNANEDIOL IN URINE

Chemical methods for the determination of pregnanediol fall into two main groups

1 Those in which pregnanediol is estimated conjugated with glucuronic acid as sodium pregnanediol glucuronide or *NaPG*
2 Those in which free pregnanediol is estimated after hydrolysis of the glucuronide complex by acid or by enzymes Methods for estimating free pregnanediol can be further subdivided into (*a*) precipitation methods and (*b*) chromatographic methods

### 1 Estimation of 'Conjugated' Pregnanediol

(*a*) THE METHOD OF VENNING (1937, 1938) —The main steps in this procedure are as follows (1) extraction of the urine with butanol (2) distribution of the butanol soluble material between butanol and aqueous NaOH (3) precipitation of the material in the water washed butanolic phase from aqueous solution by acetone (4) weighing of the precipitate formed The latter is assumed to consist largely of sodium pregnanediol glucuronide

The Venning method has been widely used in the clinical field and has yielded much valuable information It has however certain important limitations which should be recognised by those employing it In the first place stringent precautions must be taken to avoid growth of bacteria in urine as this may cause conversion of the conjugated to free pregnanediol if the urine is not suitably preserved and adequately refrigerated after collection falsely low readings for NaPG will be obtained Secondly, although the method

(*d*) extraction of progesterone from the water diluted methanolic solution by petroleum ether, (*e*) separation of progesterone by paper chromatography, (*f*) final determination of the hormone by means of ultra violet spectroscopy

The accuracy of the method was tested in a series of recovery experiments in which known amounts of progesterone were added to blood The mean recovery figure obtained was approximately 80 per cent As little as 0 05 μg of progesterone per ml of blood could be detected The mean concentration of blood progesterone in women during the second half of pregnancy was 0 078 μg per ml while in women during the normal menstrual cycle the concentration was generally below 0 05 μg per ml It will be noted that these figures are considerably lower than those obtained by the Hooker Forbes test This finding supports the view that the latter method is less specific than was originally supposed

Zander (1954) has been able to detect the presence of progesterone by chemical means in human placental tissue, in corpora lutea in corpus luteum cysts and in follicular fluid He also showed that, following the intravenous administration of progesterone to ovariectomised and post menopausal women, the blood level of the hormone rose significantly and attained a maximum some three to five minutes after the injection Subsequently a rapid fall in the titre occurred and within twenty four hours the substance could no longer be detected in the peripheral blood

The introduction of a reliable chemical method for the determination of the progesterone content of blood and tissues is an event of considerable importance The technique of Zander and Simmer is probably not suitable for routine use but should be of value in research centres Estimations by this method in normal and pathological conditions in man will be awaited with interest and such studies should yield important information in the fields of obstetrics, gynæcology and internal medicine

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yields reasonably satisfactory results when quantities greater than 15 mg of pregnanediol per twenty four hours are being measured, the procedure is unreliable in urine specimens containing smaller concentrations of pregnanediol For this reason results obtained during the normal menstrual cycle and in early pregnancy should be accepted with some reserve Finally it has been shown that the apparently purified final product of NaPG is contaminated with glucuronides of sub stances other than pregnanediol Marrian and Gough (1946) demonstrated that the NaPG obtained from pregnancy urine by the Venning method contained approximately 20 per cent of a water soluble derivative of 5$\beta$ pregnan 3$\alpha$ ol 20 one, and in the following year Sutherland and Marrian (1947) identified this contaminating substance as sodium pregnanolone glucuronide

(*b*) MODIFICATIONS OF THE VENNING METHOD —Numerous modifications of the Venning method have been proposed, but in general these procedures are open to the same objections as is the original technique Westphal (1944) precipitated pregnanediol glucuronide from urine extracts by means of barium acetate He found that the barium salt of pregnanediol glucuronide was more insoluble than the sodium salt and concluded that the precipitation was probably more complete than in the original Venning procedure Various investi gators have attempted to assay pregnanediol by measuring the glucuronic acid liberated from the NaPG after hydrolysis by acid In the technique described by Allen and Viergiver (1941) pregnanediol glucuronide was precipitated as its lead salt and the reducing power of the precipitate was estimated by means of an alkaline copper reagent Acid hydrolysis was carried out and the reducing power was re estimated The difference in reducing power before and after hydrolysis was considered to provide a measurement of the glucuronic acid content of the precipitate Crismer (1939), Jayle *et al* (1943 *a*, *b*) and Bissett *et al* (1948) determined the glucuronic acid content of NaPG by means of a colour reaction with Tollens naphtho resorcinol reagent This reaction has a high degree of sensitivity but is probably relatively non specific

Jayle and his collaborators (Jayle, 1950 Jayle *et al*, 1949 Libert, 1950) have published a long series of papers in which they claim that, by extracting urine at varying *p*H s with butanol, different groups of steroid glucuronides can be

determined The procedure has been termed a butylogram The final step in the method is a colorimetric estimation of glucuronic acid Much of the data presented deals with fractions extracted from urine at $pH$ 13 such fractions are stated to consist mainly of pregnanediol glucuronide The urinary glucuronide excretion has been studied in a large number of clinical conditions in man and attempts have been made to correlate the glucuronide excretion with the clinical features of certain disease states The methods of Jayle and his associates have been used by investigators in France but have not found favour with workers in most other countries The terminology employed is complex and specialised and the procedures themselves have many disadvantages from the quantitative point of view In the opinion of the author the work is outside the scope of the pre ent book For further information the reader is referred to the original articles

## 2 Estimation of 'Free' Pregnanediol

### (a) Precipitation Methods

(1) THE ASTWOOD TALBOT PROCEDURE —In 1941 Astwood and Jones described a new method for the quantitative determination of urinary pregnanediol which avoided certain of the disadvantages of the original Venning procedure The main steps in this method were as follows (i) hydrolysis of the urine by boiling with hydrochloric acid (ii) extraction by toluene (iii) removal of acidic and phenolic substances from the extract by treatment with NaOH (iv) purification of the pregnanediol in the neutral toluene soluble fraction so obtained by repeated precipitation from alcoholic solution by 0 1 N NaOH and water (v) weighing of the final product

This method represented one of the first attempts to estimate urinary pregnanediol in the free instead of in the conjugated form However the accuracy of the technique was not high and frequently losses of pregnanediol amounted to 20 per cent or more The sensitivity of the procedure was increased by Talbot *et al* (1941) who determined the finally purified pregnanediol not by weighing but by means of the yellow colour it yields with concentrated sulphuric acid

This colour reaction with minor modifications has been employed by most investigators who have subsequently

developed assay methods for pregnanediol It must be emphasised that the reaction itself is by no means specific for this steroid and accordingly the specificity of methods based on it depends entirely on the purity and identity of the final product obtained It is, for example, well known that a considerable over estimate of 'apparent' pregnanediol will occur if the final extract is contaminated with urinary pigments, with certain steroids other than pregnanediol, particularly decomposition products of 5$\beta$ pregnane 3$\alpha$ 17$\alpha$ 20$\alpha$ triol, and also perhaps with non steroidal colourless substances For a more detailed account of the various factors influencing the sulphuric acid colour reaction the reader is referred to a recent article by Klopper (1956 *a*)

(2) THE METHOD OF GUTERMAN AND SCHROEDER (1948) —This is merely a simplified and shortened version of the Astwood Talbot procedure by means of which pregnanediol determinations can be completed in a few hours The technique has been used as a method of diagnosing pregnancy It is, however, probable that the results obtained by this procedure have little significance from the quantitative point of view

(3) THE METHOD OF SOMMERVILLE, GOUGH AND MARRIAN (1948 *a*) —This method is also a modification of the Astwood Talbot procedure and will measure with reasonable accuracy (ca 82 per cent) approximately 2 mg of pregnanediol per twenty four hour urine specimen At lower levels of excretion however, its accuracy is unsatisfactory Furthermore, the procedure is laborious and time consuming and its specificity is somewhat questionable In view of the development of better techniques for the assay of urinary pregnanediol such as that recently published by Klopper *et al* (1955) it is unlikely that this procedure will continue to be used in clinical studies

(4) THE METHOD OF SOMMERVILLE MARRIAN AND KELLAR (1948 *b*) —This is a more rapid and less elaborate version of method (3) The original authors claimed that the technique could measure pregnanediol levels of 5 mg per twenty four hours or more and that it was therefore suitable for routine estimations in mid and late pregnancy Subsequent work has, however, demonstrated that the procedure is inaccurate and relatively non specific and it is doubtful if the results obtained by its use have much quantitative significance

### (*b*) Chromatographic Methods

A number of techniques have been proposed in which urinary pregnanediol is purified by methods depending on chromatography The most satisfactory procedures from the clinical point of view are those described by de Watteville Borth and Gsell (1948) and by Klopper Michie and Brown (1955)

(1) The Method of de Watteville, Borth and Gsell (1948) —This procedure is based on a method originally described by Huber (1947) and is essentially a modification of the technique of Astwood and Jones (1941) The neutral steroid fraction which is obtained after hydrolysis of the glucuronide by acid is chromatographed on alumina columns subsequently the free crystalline pregnanediol obtained by elution with a benzene alcohol mixture, is weighed Much clinically useful information has been obtained by the use of this technique in normal and pathological conditions The method is, however rather less sensitive and somewhat less specific than that recently developed by Klopper *et al* (1955)

(2) The Method of Klopper Michie and Brown (1955) —This method which includes several novel features is probably superior in all respects to any that have been previously described The main steps in the procedure are (i) acid hydrolysis (ii) toluene extraction, (iii) a new per manganate oxidation step designed to remove from the pregnanediol fraction a contaminant which is probably a decomposition product of pregnanetriol (iv) chromatography on alumina columns (v) acetylation (vi) further chromato graphy on alumina (vii) colorimetry by means of a modified version of the usual sulphuric acid reaction By infra red spectroscopy and by melting point and mixed melting point determinations it has been shown that the finally purified product contains $5\beta$ pregnane $3\alpha$ $20\alpha$ diol diacetate and virtually nothing else (Klopper *et al* 1955 Coyle *et al*, 1956) Accordingly it can be claimed that the method is highly specific for $5\beta$ pregnane $3\alpha$ $20\alpha$ diol

The accuracy of the procedure was tested in a series of recovery experiments in which known amounts of pure pregnanediol were added to portions of twenty four hour urine samples obtained from non pregnant subjects At concentra tions of the steroid as low as 0 5 mg per twenty four hours

the mean recovery of added pregnanediol was approximately 92 per cent, at higher concentrations of pregnanediol the mean recovery figures were even better

Typical pregnanediol values obtained from the urine of men and of women during the menstrual cycle and after the menopause are shown in Table XVI

TABLE XVI

PREGNANEDIOL CONTENT OF TWENTY FOUR HOUR URINES FROM VARIOUS SOURCES

*(From Klopper et al 1955)*

| Type of Urine | Number of Individuals | Number of Determinations | Range mg per Twenty four Hours | Average Excretion mg per Twenty four Hours |
|---|---|---|---|---|
| Male | 9 | 50 | 0 38 to 1 42 | 0 92 |
| Female proliferative phase | 4 | 8 | 0 78 to 1 50 | 1 12 |
| Female luteal phase | 2 | 18 | 2 1 to 4 2 | 3 30 |
| Female post menopausal | 5 | 23 | 0 28 to 0 86 | 0 63 |

It will be noted that the technique is sufficiently sensitive to measure 'adrenal' as well as 'ovarian pregnanediol' The significance of this finding will be discussed later in this chapter

The method is less exacting and time consuming than many of the previously published techniques, and it is probably suitable for routine application in the clinical field One technician can complete approximately twenty estimations in one week

## THE CLINICAL SIGNIFICANCE OF URINARY PREGNANEDIOL ESTIMATIONS

One of the main reasons for performing urinary pregnanediol determinations in the clinical field is to obtain information regarding the production of endogenous progesterone by the body Accordingly it is necessary to consider the quantitative relationship between the secretion of progesterone on the one hand and the excretion of urinary pregnanediol on the other

Information pertaining to this problem can be accumulated by studying the relationship of the blood progesterone concentration to the urinary pregnanediol excretion by measuring the urinary pregnanediol output following the administration of progesterone in normal and disease states and by investigating the role of various organs, particularly the liver in the conversion of progesterone to pregnanediol

It is generally agreed that the concentration of progesterone in human blood is very low This is the case whether the hormone is assayed by biological or by chemical methods The most reliable estimates of blood progesterone at present available are those of Zander (1954) who reported mean concentrations of less than 0 1 µg per ml in the second half of pregnancy and of less than 0 05 µg per ml during the luteal phase of the normal menstrual cycle On the other hand pregnanediol, which is the principal urinary metabolite of progesterone, is excreted in relatively large quantities, in late pregnancy figures varying from 40 to 80 mg per twenty four hours are frequently encountered and in the luteal phase of the menstrual cycle the excretion values generally lie between 2 and 5 mg per twenty four hours These two sets of observations taken in conjunction support the view that pro gesterone is rapidly metabolised in the body and this has indeed been found to be so when the hormone has been administered intravenously to human subjects and to animals In such experiments progesterone was shown to disappear very rapidly from the circulation and could no longer be detected in the blood a few hours after the injection

Much has been written regarding the urinary excretion of pregnanediol in human subjects following the administration of progesterone Some of the early work in this field is probably of little significance as the methods of estimation of urinary pregnanediol were of doubtful reliability at the low levels of excretion often encountered in such metabolic studies More recent investigations with improved methods of assay have shown that in males and in post menopausal females less than 20 per cent of the dose of administered progesterone is excreted in the urine as pregnanediol This observation merits emphasis in view of the widely held assumption that the urinary pregnanediol excretion is a reasonably exact index of the endogenous progesterone production

A considerable body of experimental evidence indicates that the liver is intimately concerned with progesterone metabolism and that therefore this organ is capable of influencing the urinary pregnanediol excretion in health and disease This evidence, some of which will be briefly considered below, has led to the conclusion that the liver is the organ mainly responsible not only for the reduction of progesterone to pregnanediol but also for the conjugation of pregnanediol with glucuronic acid

Many investigators have demonstrated that liver slices can metabolise progesterone and have studied the enzyme systems responsible for this effect Pellets of progesterone implanted into the spleen or mesentery of rabbits rapidly lose their biological activity and this finding supports the view that the liver is the main site of progesterone inactivation in the body Riegel *et al* (1950) administered progesterone labelled with $^{14}C$ to rats and mice and found that relatively large amounts of radio activity appeared in the liver and in the fæces Grady *et al* (1952) showed that in rats radio active progesterone passed from the liver to the intestine via the common bile duct and that complete biliary obstruction resulted in the appearance of considerable quantities of radio activity in the urine Rogers and McLellan (1951) and Klopper (1956 *b*) have detected the presence of pregnanediol in human bile following the administration of progesterone to patients in whom biliary drainage had been established Rogers (1956) estimated the urinary pregnanediol output after the administration of progesterone to patients with biliary obstruction and found that in such individuals abnormally large quantities of pregnanediol were excreted He concluded that when the biliary tract is obstructed, the route of excretion of pregnanediol is altered and a greater proportion of the steroid appears in the urine than under normal conditions This interesting observation requires confirmation

The preceding discussion has emphasised that urinary pregnanediol estimations cannot provide the clinician with a quantitative measurement of endogenous progesterone production However, there can be no doubt that the pregnanediol excretion does to some extent at least reflect the output of the hormone by the ovary or the placenta It is, for example well established that the excretion of urinary pregnanediol is

generally high in cases in whom it would be reasonable to expect that the progesterone production is relatively high and that the output is frequently low in patients who show evidence of luteal deficiency or placental failure In view of these observations it would appear justifiable to conclude that clinically useful information can often be obtained by preg nanediol assays in urine although the exact significance of such determinations remains a problem for future elucidation

## THE URINARY EXCRETION OF PREGNANEDIOL IN NORMAL SUBJECTS

### 1 Children

Bergstrand and Gemzell (1957) have recently applied the assay method described by Klopper *et al* (1955) to a study of the urinary *pregnanediol excretion in eighty seven normal* children between the ages of three and fifteen They found that in boys the mean pregnanediol output was 0 75 mg per twenty four hours while in girls the corresponding figure was 0 72 mg per twenty four hours No correlation could be demonstrated between the pregnanediol excretion on the one hand and the age of the children on the other

### 2 Normally Menstruating Women

The urinary excretion of pregnanediol during the normal menstrual cycle has been studied by numerous investigators over the past two decades In the earlier investigations the method of pregnanediol assay described by Venning (1937) was used By means of this technique urinary pregnanediol could be demonstrated in the luteal but not in the follicular phase of the cycle It was also shown that a rapid fall in the pregnanediol excretion occurred one or two days prior to the onset of menstruation Values obtained from normal women at the same stage of the cycle showed a very wide scatter from one subject to another but the average excretion during the luteal phase was approximately 5 mg of NaPG per twenty four hours

One of the more careful studies of the urinary pregnanediol excretion in normally menstruating women is that of de Watteville (1951) who used the technique of pregnanediol assay described by de Watteville *et al* (1948) This method

which depends on chromatography, is probably more specific than that of Venning, and for this reason the figures for the pregnanediol excretion are somewhat lower than those obtained by the Venning method De Watteville (1951) found that the excretion of pregnanediol in individual cases varied greatly from one day to another and did not follow any definite pattern There was also considerable variation in excretion from one subject to another at comparable stages of the cycle In a small proportion of apparently healthy women pregnanediol

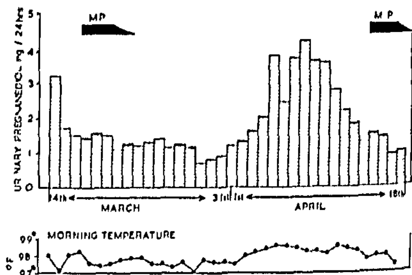



Fig 47

Urinary excretion of pregnanediol in a typical menstrual cycle Basal temperature readings are also shown M P = menstrual period (*From Klopper, 1957*)

could not be detected at any time during the cycle This worker wisely emphasised that the trend in a series of pregnanediol determinations *in the same patient gives much more* information to the clinician than isolated readings taken at widely separated intervals

The development by Klopper *et al* (1955) of a more sensitive procedure for the quantitative measurement of urinary pregnanediol has made it possible to estimate this steroid in urine during the whole of the normal menstrual cycle Typical results obtained by this method in individual cases are shown in Figs 47, 48 and 49 which are taken from a paper by Klopper (1957)

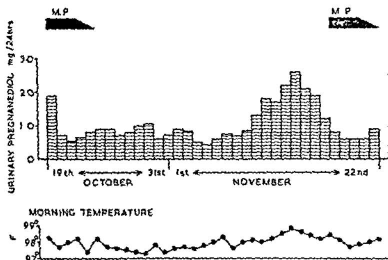



Fig 48

Urinary excretion of pregnanediol in a normal menstrual cycle—juvenile type Basal temperature readings are also shown M P = menstrual period (*From Klopper* 1957)

Fig 49

Urinary excretion of pregnanediol in a normal menstrual cycle—adult type Basal temperature readings are also shown M P = menstrual period (*From Klopper* 1957)

It will be noted that in the follicular phase of the cycle the excretion of pregnanediol is usually 1 mg per twenty four hours or less, this material presumably represents mainly adrenal pregnanediol' which is derived from precursors secreted by the adrenal cortex At the time of ovulation a sharp rise in the pregnanediol excretion occurs During the luteal phase the values generally lie between 2 and 5 mg per twenty four hours, at this time the pregnanediol is almost certainly derived from the progesterone secreted by the luteal tissue of the ovary The pregnanediol output begins to fall several days before the onset of menstruation and continues to decline for the first two or three days after the onset of the period

## 3 Normal Men

Small but measurable quantities of adrenal pregnanediol are present in male urine In a series of fifty determinations in nine normal male subjects Klopper *et al* (1955) found that the mean excretion was 0 92 mg per twenty four hours and that the levels ranged from 0 38 to 1 42 mg per twenty four hours

## 4 Post-menopausal Women

In patients in this age group the pregnanediol excretion is usually low The steroid is probably derived to a large extent from precursors secreted by the adrenal cortex In a series of twenty three determinations in five post menopausal women, Klopper *et al* (1955) found that the mean excretion was 0 63 mg per twenty four hours and that the readings varied from 0 38 to 0 86 mg per twenty four hours

## 5 Normal Pregnancy and Labour

The urinary excretion of pregnanediol during normal pregnancy has been estimated by many workers including Browne *et al* (1937) Venning (1938) Bachman *et al* (1941), Jones *et al* (1944), Plotz and Darup (1950) and Michie (1953) In the earlier investigations the Venning method of determination was employed and the steroid was estimated in the conjugated form as NaPG, in later studies estimates were made by techniques which measured the urinary pregnanediol in the free' rather than the conjugated state

The original curve for urinary NaPG during pregnancy as published by Venning (1938) is shown in Fig 50

It will be noted that the pregnanediol excretion gradually increases as pregnancy advances At the twelfth week the readings generally lie between 10 and 15 mg per twenty four hours By the twentieth week the excretion averages 30 mg per twenty four hours and by the thirty sixth week the output

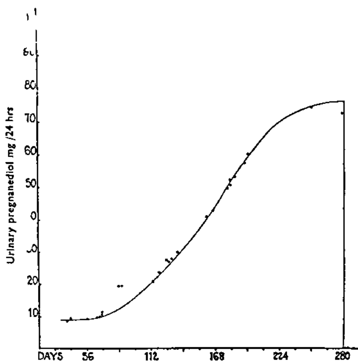



Fig 50

Urinary excretion of pregnanediol during normal pregnancy in eight subjects (*From Venning* 1938)

is approximately 80 mg per twenty four hours From the thirty sixth week onwards most workers have found that the mean excretion of urinary pregnanediol does not show a further rise and may even decrease slightly No definite relationship has so far been demonstrated between the output of pregnanediol on the one hand and the onset of labour on the other After delivery the amount of pregnanediol in the urine decreases rapidly and by the first few days of the puerperium

the levels have fallen to those normally encountered in non pregnant individuals

Curves of the same general shape as that obtained by Venning (1938) have been published by later workers who determined urinary pregnanediol in the 'free' rather than the conjugated form Among the more detailed of such studies are those of Jones *et al* (1944) who used the method of Astwood and Jones (1941) and of Bradshaw and Jessop (1953) and Michie (1953) who employed the technique described by

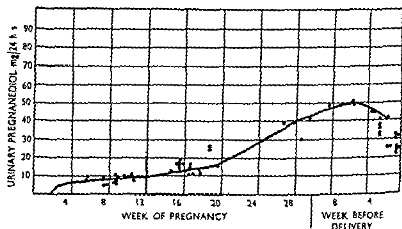



Fig 51

Urinary excretion of pregnanediol during normal pregnancy Scatter diagram of 87 determinations in 52 cases The values for the last three months are plotted backwards from the date of delivery The curve connects monthly means (*From de Watteville* 1951)

Sommerville *et al* (1948 *b*) The curve obtained by de Watteville (1951) using the assay method of de Watteville *et al* (1948) is shown in Fig 51

It is probable that the pregnanediol output reflects to some extent the growth and development of the placenta The excretion of the steroid starts to rise when the placenta is formed it increases progressively during the period of active proliferation of the cytotrophoblast and it remains relatively constant after the thirty sixth week by which time placental growth is virtually complete

All the work published so far has indicated that the urinary pregnanediol excretion varies greatly from one subject to another at comparable stages of pregnancy and that large variations also occur in the daily excretion of the steroid in

the same individual Accordingly, it is unlikely that the clinician will derive much benefit from data based on single determinations of pregnanediol in individual cases or on isolated readings made at widely separated intervals during pregnancy in the same patient Only by studying the trend in a series of pregnanediol determinations in individual cases will it be possible to obtain information of clinical importance

Most investigators who have published curves for the urinary excretion of pregnanediol during normal pregnancy have employed assay methods of doubtful accuracy and specificity such as those developed by Venning (1938) Astwood and Jones (1941) and Sommerville *et al* (1948 *b*) Although valuable clinical information has been obtained by the use of such techniques it is doubtful whether the results reported have much significance from the quantitative point of view Recently Coyle *et al* (1956) employed the assay method described by Klopper *et al* (1955) in a study of the urinary excretion of pregnanediol in a small series of normally pregnant women and it is probable that the results which these workers obtained are more reliable than any previously published They found that the pregnanediol levels during normal pregnancy were lower than those reported by previous investigators this observation probably reflects the greater specificity of the assay method used During the gestation period a gradual rise in pregnanediol output was noted until about the thirty second week when the mean excretion was approximately 40 mg per twenty four hours From the thirty second week until term no further increase in pregnanediol excretion occurred After delivery the output fell rapidly and in less than one week levels of 2 to 3 mg per twenty four hours were observed

### Urinary Pregnanediol Assay as a Means of Pregnancy Diagnosis

Guterman (1947) attempted to use pregnanediol estimations for this purpose He developed a simple and rapid semi quantitative method of determination by the use of which results were obtained in a few hours The Guterman test has been severely criticised by many workers and should no longer be used as a method of pregnancy diagnosis One of the main disadvantages of the test is the fact that values obtained in early pregnancy are often quantitatively similar to those

found during the luteal phase of the normal menstrual cycle The subject of pregnancy diagnosis is considered in detail in Chapter III

## THE EXCRETION OF URINARY PREGNANEDIOL IN PATHOLOGICAL CONDITIONS

### 1 Disorders of Menstruation

(*a*) ANOVULAR MENSTRUATION —In this condition cyclic uterine bleeding occurs in the absence of ovulation and corpus luteum formation The uterine bleeding therefore takes place from a proliferative and not from a secretory endometrium Anovulatory cycles probably occur relatively frequently in the human female but are more common at the beginning and towards the end of reproductive life For further information on the clinical features of anovular menstruation the reader is referred to Chapter IX

Many workers over the past two decades have demonstrated that the pattern of excretion of urinary pregnanediol in normally menstruating women differs from that found in patients with anovular menstruation In the former group the steroid was not detected in the urine at any time during the follicular phase of the cycle, but appeared in relatively large quantities during the luteal phase, in the latter group, on the other hand pregnanediol was shown to be absent from the urine during the whole cycle These findings taken in conjunction form the basis of the widely held belief that urinary pregnanediol assays are of considerable clinical value in determining the time of ovulation in women and in differentiating ovular from anovular menstruation However, recent developments in the field of pregnanediol assay have suggested that these views may have to be modified and that urinary pregnanediol estimations may be of rather less diagnostic value than was previously supposed in cases of suspected anovular menstruation

In the majority of the investigations in which the pregnanediol excretion in ovular and anovular menstruation were compared, relatively insensitive assay methods such as those of Venning (1938) or of de Watteville *et al* (1948) were employed Such techniques were probably not capable of detecting the steroid at concentrations of less than 2 mg per twenty four hours Recently Klopper *et al* (1955) have developed a more

sensitive assay procedure for pregnanediol which will estimate with reasonable accuracy as little as 0 5 mg in a twenty four hour specimen of urine This technique is sufficiently sensitive to detect the presence of the steroid in the follicular as well as the luteal phase of the normal menstrual cycle (Figs 47 to 49) During the follicular phase the urinary pregnanediol is probably derived to a large extent from precursors secreted by the adrenal cortex and may be termed adrenal' pregnanediol Although studies of pregnanediol excretion in anovular menstruation using this assay method have not yet been published, it is reasonable to assume that in such patients also adrenal pregnanediol will be demonstrated in the urine

These recent findings lead to the conclusion that the mere presence of pregnanediol in the urine is not of itself evidence that ovulation has occurred Accordingly it would appear that urinary pregnanediol determinations can only be of limited diagnostic value in cases of suspected anovular menstruation In this type of case such estimations should be regarded as complementary to other more routine methods of assessing corpus luteum function such as those depending on basal temperature curves on vaginal smears or on endometrial biopsy

(*b*) AMENORRHŒA —In cases of both primary and secondary amenorrhœa the pregnanediol excretion is low, being generally below 1 mg per twenty four hours (Klopper 1956 *b*) It is probable that the material present represents adrenal rather than ovarian pregnanediol

(*c*) PERSISTENT CORPUS LUTEUM —This is a rare condition of unknown ætiology The corpus luteum is cystic and resembles very closely the corpus luteum of early pregnancy The endometrium shows advanced secretory changes Amenorrhœa is generally present and it is believed that *menstruation is prevented by the presence in the circulation* of large quantities of progesterone In the few cases of persistent corpus luteum so far reported in the literature hormone assays by reliable methods have not been conducted

(*d*) IRREGULAR SHEDDING OF THE ENDOMETRIUM —This condition was first described by Pankow in 1924 The characteristic feature of the disease is the fact that the endometrium is shed over an abnormally long period of time Histological examination of the endometrium obtained during the bleeding

phase shows the presence of secretory changes Clinically the condition is characterised by prolonged and excessive bleeding at the time of menstruation The cycles themselves are not increased in length

McKelvey and Samuels (1947) demonstrated that pregnanediol measured as NaPG was excreted in the urine throughout the period of uterine bleeding These workers suggested that an ætiological factor in this disease might be delayed involution of the corpus luteum Further hormone estimations in patients with this condition will be awaited with interest

(*e*) STEIN LEVENTHAL SYNDROME—The main clinical features of this relatively rare disease are cystic enlargement of both ovaries secondary amenorrhœa, sterility and various stigmata of masculinisation of which hirsutism is the most prominent The characteristic finding on histological examination of the ovaries is hyperplasia of the cells of the theca interna For a detailed account of the clinical and pathological features of the Stein Leventhal syndrome the reader is referred to a recent article by Shippel (1955)

Numerous theories have been advanced to explain the virilisation which is frequently found in this disease Leventhal and Cohen (1951) considered that this might be caused by excessive production of progesterone by the theca cells On the other hand Shippel (1955) believes that the theca cells are capable of elaborating an androgenic hormone which is responsible for the masculinising features of the syndrome

At the time of writing urinary pregnanediol assays have been performed in relatively few patients with the Stein Leventhal syndrome In the case studied by Fischer and Riley (1952) abnormally large quantities of this steroid were excreted but in a patient recently investigated in Edinburgh by Klopper (1956 *b*) the pregnanediol output was within the normal range

## 2 Abnormal Pregnancy

(*a*) THREATENED AND RECURRENT ABORTION—Views are conflicting with respect to the clinical value of urinary pregnanediol assays in patients with these conditions The subject has recently been reviewed by Borth and de Watteville (1952) Although several investigators have demonstrated subnormal

values preceding abortion cases are frequently encountered in which interruption of pregnancy occurs in the presence of normal pregnanediol values or fails to occur despite abnormally low values Findings of this type are not surprising in view of the multiplicity of causes of abortion among which progesterone deficiency is probably by no means the most frequent After three years of experience of routine pregnanediol assays carried out in the laboratory of the Simpson Memorial Maternity Pavilion in Edinburgh Kellar (1952) came to the conclusion that the test was of little diagnostic value in cases of threatened and recurrent abortion This worker found that in cases in which a falling pregnanediol excretion was observed, there were generally very obvious clinical signs of the inevitability of a threatened abortion A similar conclusion was reached by Cope (1940) Swyer (1949) and others

Guterman (1950) believes that the trend of the urinary pregnanediol excretion may sometimes be of prognostic value in cases of threatened abortion In his series of cases he found that abortion occurred frequently in patients who showed a progressive fall in pregnanediol excretion to levels abnormally low for the period of gestation He also demonstrated that abortion was relatively rare in patients in whom the pregnanediol excretion was within the normal range or had risen to normal from an initially low level De Watteville (1951) emphasised that single readings in individual patients or isolated pregnanediol determinations made at widely separated intervals in the same patient were of virtually no diagnostic value He did however suggest that clinically useful information could sometimes be obtained by following the trend in a series of urinary pregnanediol determinations in selected cases After a careful and critical review of the literature Borth and de Watteville (1952) concluded that in cases of threatened abortion and premature labour, serial estimations of urinary pregnanediol may sometimes be of value in assessing the functional state of the trophoblast In the opinion of these workers fœtal death is generally associated with decreasing or consistently low levels of urinary pregnanediol Furthermore they believe that abnormally low values occurring in early pregnancy in patients with a history of repeated abortions may precede any clinical sign of abortion and may justify preventive therapy by means of progesterone

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It is obvious that, at the time of writing, there is much controversy regarding the clinical value of pregnanediol assays in patients with threatened and recurrent abortion Further work is necessary before any definite conclusions can be drawn

(*b*) PRE ECLAMPTIC TOXÆMIA —An abnormally low excretion of urinary pregnanediol in a proportion of toxæmic patients has been reported by numerous investigators including Browne *et al* (1938), Smith and Smith (1938) and de Watteville (1951) There appears to be little or no correlation between

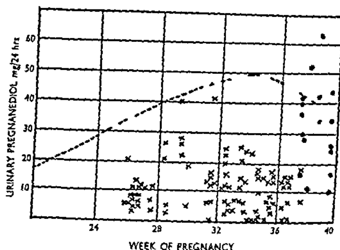



FIG 52

Urinary excretion of pregnanediol in patients with pre-eclamptic toxæmia Closed circles denote normal child crosses denote dead or under-developed child The dotted line is the mean curve shown in Fig 51 (*From de Watteville* 1951 )

the pregnanediol output on the one hand and any clinical feature of the disease, such as hypertension, œdema or albuminuria, on the other De Watteville (1951), in a careful study, reported that the excretion varied greatly from one subject to another but found that the majority of estimations lay below the normal range He divided his cases into two groups In the first group consisting of patients who were delivered of normal babies the pregnanediol excretion was generally within normal limits The second group consisted of cases in which the child was born dead or under developed and in these patients almost all the readings obtained were abnormally low De Watteville s results are shown in Fig 52

The cause of the low pregnanediol excretion in pre eclamptic toxæmia is not known with certainty It could be due to impaired placental function as a result of spasm of the spiral arterioles in the decidua to alterations in the metabolism of progesterone, or to abnormalities in the renal clearance of the hormone Smith and Smith (1940 1948) were among the first to note the low pregnanediol excretion in cases of pre eclamptic toxæmia, and these workers attempted to weave this finding into a complicated pattern of ætiology of pre eclampsia The Smith theories, although stimulating and provocative have never been substantiated and are not now generally accepted as valid For further information on these theories the reader is referred to the original articles

(c) DIABETIC PREGNANCY —White and her collaborator (White, 1947, 1952, White and Hunt 1943) have stated that a relatively high proportion of pregnant diabetics show an abnormally low excretion of urinary pregnanediol and have claimed that this finding is of diagnostic and prognostic value in relation to the fate of the fœtus In the experience of those investigators many patients showed the condition of hormonal imbalance which was characterised by abnormally low levels of urinary pregnanediol and serum œstrogens and by abnormally high concentrations of serum HCG In such patients the incidence of obstetrical complications was much higher than in those in whom the hormonal balance was normal Correction of the hormonal imbalance by substitution therapy by œstrogens and progesterone was said to restore the hormone readings to normal and to improve greatly the prognosis for the fœtus

Other workers have been unable to confirm White s findings in relation to urinary pregnanediol estimations in cases of pregnancy complicated by diabetes Keltz *et al* (1950) noted that the mean figure for pregnanediol excretion in the third trimester of pregnancy was significantly higher in patients delivering a viable fœtus than in those in whom fœtal death occurred However these investigators were unable to convince themselves that pregnanediol assays were of any diagnostic or prognostic value in pregnant diabetics A similar conclusion was reached by Michie (1954) after a careful study conducted in Edinburgh It should be emphasised that pre eclamptic toxæmia may frequently co exist with

diabetic pregnancy This is of some importance in relation to urinary pregnanediol assays as the former condition *per se* may markedly influence the urinary output of this steroid The subject of hormone assays in pregnant diabetics is further discussed in Chapter III

## 3 Adrenal Disease

Urinary pregnanediol assays in patients with adrenal hyperplasia and adrenal tumours present considerable difficulties owing to the presence in the urine of large quantities of 17 ketosteroids and 5β pregnane 3α 17α 20α triol These substances when they occur in high concentration may interfere to a considerable extent with the specificity of colorimetric assay methods for urinary pregnanediol Accordingly, figures for the excretion of ' pregnanediol in such cases should be viewed with some reserve unless evidence has been produced that the steroid has actually been isolated and identified by chemical means

Various investigators, including Malley and Bradshaw (1941), Talbot *et al* (1942) and Lloyd *et al* (1951) have claimed that patients with adrenal hyperplasia and adrenal tumours excrete abnormally large quantities of urinary pregnanediol The methods of assay used by these workers were relatively non specific, and it is doubtful if the results they obtained had much quantitative significance Bongiovanni *et al* (1954) have shown that, although pregnanediol excretion may sometimes be increased in cases of adrenocortical hyperfunction this increase is usually overshadowed by the presence in the urine of abnormally large amounts of pregnanetriol In view of this observation it is quite possible that the pregnanediol estimated by previous investigators consisted largely of decomposition products of pregnanetriol which had been formed after acid hydrolysis As emphasised by Klopper *et al* (1957) the presence of abnormally high levels of urinary pregnanediol in cases of adrenocortical hyperfunction cannot be definitely established until methods of assay have been applied which are capable of separating urinary pregnanediol from large amounts of other steroids such as pregnanetriol pregnanolone and androstanediol

URINARY PREGNANEDIOL ESTIMATIONS AS A TEST OF ADRENOCORTICAL FUNCTION IN MAN—Klopper *et al* (1957) have recently studied the effect of ACTH administration and of

bilateral adrenalectomy on the urinary excretion of preg nanediol They found that the intravenous infusion of ACTH at a rate of one international unit per hour caused a sharp rise in urinary pregnanediol output and that a similar effect was produced by operative stress Results in a healthy male subject before and after ACTH administration are shown in Fig 53 It will be noted that in this subject ACTH produced a fourfold increase in urinary pregnanediol excretion

After bilateral adrenalectomy on the other hand, Klopper

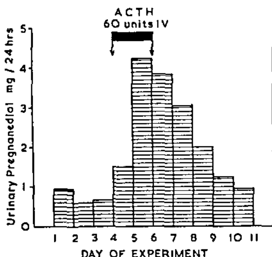



FIG 53

The effect of ACTH on the urinary excretion of pregna nediol in a normal male subject (*From Klopper et al* 1957)

*et al* (1957) and Strong *et al* (1956) showed that the urinary pregnanediol output fell to very low levels The intravenous administration of ACTH to adrenalectomised patients failed to produce any rise in pregnanediol output

On the basis of these observations Klopper *et al* (1957) have suggested that urinary pregnanediol assays may be of value as a means of assessing adrenocortical function in man The test appears to offer an alternative to such well recognised methods of estimating adrenocortical activity as eosinophil counts water excretion tests electrolyte studies, the estimation of 17 ketosteroids in urine and the determination of 17 21 dihydroxy 20 ketosteroids in blood and in urine

## THE EXCRETION OF URINARY PREGNANEDIOL FOLLOWING PROGESTERONE ADMINISTRATION IN NORMAL AND DISEASE STATES

### 1 Normal Non pregnant Subjects

Many investigators have accumulated information on the urinary excretion of pregnanediol in men and women following the administration of progesterone Much of the early work in this field was performed with methods which were of doubtful accuracy at the low levels of pregnanediol excretion often encountered in such metabolic experiments It is therefore

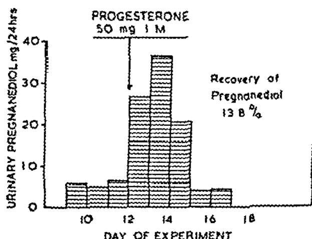



Fig 54
A typical recovery experiment in a post menopausal subject
(*From Klopper* 1956 *b*)

not surprising that the amounts of progesterone recovered a urinary pregnanediol varied greatly from one laboratory t another More recent studies with superior methods have demonstrated that in men and post menopausal women the proportion of administered progesterone excreted as urinary pregnanediol generally ranges from 10 to 20 per cent A typical experiment in a post menopausal subject in which the assay method of Klopper *et al* (1955) was used is shown in Fig 54

According to Fischer and McColgan (1953) the conversion rate of progesterone to pregnanediol is not affected by the mode of exhibition of the hormone Similar conversion rates

were obtained using oral, sublingual or parenteral routes of administration of standard doses of progesterone

PROGESTERONE 'PRIMING'—Sommerville and Marrian (1950) and Sommerville (1950), using the method of pregnanediol assay described by Sommerville *et al* (1948 *a*), reported that, on administering progesterone daily to intact post menopausal women for periods up to twenty seven days, two plateaux levels of pregnanediol excretion were observed The first of these was found on the second or third day, at this time the conversion rate of progesterone to pregnanediol usually lay between 10 and 20 per cent Thereafter at about the fifth to the eighth day the pregnanediol output rose in a regular stepwise manner to a second plateau level which was maintained for the duration of the experiment At the time of the second plateau the conversion rate was greater than 20 per cent This phenomenon was termed a progesterone 'priming' effect Sommerville and Marrian (1950) and Sommerville (1950) also claimed that no 'priming' effect was observed when progesterone was administered daily to normal men and to post menopausal subjects who had been subjected to hysterectomy or to hysterectomy and oophorectomy They therefore concluded that the uterus was necessary for the occurrence of progesterone priming and that this organ must therefore be concerned in the conversion of progesterone to pregnanediol

The work of Sommerville and Marrian (1950) has not been subsequently confirmed For example, Rothchild (1953) was unable to demonstrate progesterone priming in clinical conditions in which it might reasonably have been expected to occur The doubts cast on the validity of the original observation of Sommerville and Marrian (1950) led Marrian, Russell and Atherden (1954) to reinvestigate the whole problem These workers used the same assay method for urinary pregnanediol as that employed by Sommerville and Marrian (1950) and selected a similar series of experimental subjects They were unable to substantiate the previous findings Recently Klopper and Michie (1956) using the method of pregnanediol assay developed by Klopper *et al* (1955) studied the urinary excretion of pregnanediol in post menopausal women and in normal men during prolonged daily administration of progesterone They found that

post menopausal subjects did not show two plateaux of preg nanediol excretion and that the pattern of excretion of this steroid in post menopausal women did not differ in any way from that in a series of men similarly treated

It can therefore be concluded that the phenomenon which has been termed progesterone 'priming' does not exist and that the original observation of Sommerville and Marrian (1950) and of Sommerville (1950) was incorrect

**2 Pregnant Women**

Venning and Brown (1940) have claimed that during normal pregnancy the conversion rate of progesterone to pregnanediol is greater than is found in non pregnant subjects Guterman (1950) has studied the conversion of progesterone to pregnanediol in cases of habitual abortion and has attempted to correlate these studies with the clinical course of the disease He found that abortion almost always occurred in patients in whom the conversion rate was less than 13 per cent while in cases showing a conversion rate of 37 per cent or more preg nancy was usually maintained This work should be repeated using more accurate and specific methods of assay for urinary pregnanediol than were employed by Guterman in his original experiments

**3 Patients with Rheumatoid Arthritis**

Sommerville, Marrian Duthie and Sinclair (1950) and Sommerville (1950) claimed to have shown conclusively that in post menopausal women suffering from rheumatoid arthritis an abnormally high proportion of progesterone administered by the intramuscular route was excreted as urinary pregnane diol The conversion rate of progesterone to pregnanediol lay between 19 and 36 per cent in the patients with rheumatoid arthritis and between 9 and 16 per cent in the series of normal subjects There was no overlap between the two groups This finding of abnormal progesterone metabolism was thought to be of possible significance in relation to the ætiology of rheumatoid arthritis since the symptoms of that disease were known to be suppressed by cortisone a substance which bears a close chemical relationship to progesterone and may be metabolised in the body in a somewhat similar manner to progesterone

The original observations of Sommerville *et al* (1950) and of Sommerville (1950) have not been subsequently confirmed by Marrian and Atherden (1953) and by Roy, Wigzell Demers, Sinclair, Duthie Atherden and Marrian (1955) These latter two groups of investigators showed that the conversion rate of progesterone to pregnanediol did not differ significantly in patients with rheumatoid arthritis from that of normal individuals Accordingly it must be concluded that the earlier reports of Sommerville *et al* (1950) and of Sommerville (1950) were incorrect

**4 Patients with Liver Disease**

It is generally believed that the liver is the organ mainly responsible not only for the reduction of progesterone to pregnanediol but also for the conjugation of pregnanediol with glucuronic acid Various investigators, using rather unreliable assay methods, have reported that patients with chronic hepatic disease show a higher rate of conversion of progesterone to pregnanediol than do patients with normal liver function However more recent work with better assay methods has tended to show that the conversion rate may be within normal limits even in the presence of advanced hepatic disease

## THE EFFECT OF STILBŒSTROL ON THE URINARY EXCRETION OF PREGNANEDIOL IN NORMAL AND DIABETIC PREGNANCY

One of the main tenets of the theories of Smith and Smith in relation to pre eclamptic toxæmia was the interdependence of œstrogen and progesterone metabolism In 1946 Smith *et al*, using the method of assay of pregnanediol described by Venning (1937) reported that they had been able to cause a sharp rise in the urinary excretion of this steroid in a pregnant diabetic woman following the administration of stilbœstrol These workers concluded that this effect on urinary pregnanediol output was a result of an increased production of progesterone by the placenta and suggested that treatment with stilbœstrol might be of value in patients with histories of repeated obstetrical accidents during pregnancy Some doubts regarding the correctness of the experimental results of Smith

*et al* (1946) were raised when subsequent investigators including Davis and Fugo (1948) and Seitchik (1950) reported that stilbœstrol had no effect on the urinary excretion of pregnanediol in normal and abnormal pregnancy

Sommerville, Marrian and Clayton (1949), using a modified Astwood Talbot procedure, studied the urinary pregnanediol excretion in normal and diabetic pregnancy before, during and after the administration of stilbœstrol They obtained results exactly opposite to those of Smith *et al* (1946) A sharp fall in the urinary pregnanediol output followed the administration of stilbœstrol in all cases and withdrawal of the drug resulted in a rapid return to levels encountered in the control period The whole problem was recently reinvestigated in Edinburgh by Michie (1955) using the assay methods described by Sommerville *et al* (1948 *a*) and by Klopper *et al* (1955) This worker was unable to confirm the previous observations of Sommerville *et al* (1949) and concluded that the oral administration of stilbœstrol to normally pregnant women and to pregnant diabetics had no consistent effect on the excretion of urinary pregnanediol

## Summary and Conclusions

Assays of progesterone in blood should be conducted by chemical rather than by biological methods The technique recently described by Zander and Simmer (1954) is probably suitable for use in research laboratories and should in the future provide information of clinical importance

For the assay of pregnanediol in urine the method described by Klopper *et al* (1955) is recommended This procedure will measure with reasonable accuracy as little as 0 5 mg of pregnanediol in a twenty four hour specimen Satisfactory methods are not yet available for the quantitative determination of other progesterone metabolites in urine

Urinary pregnanediol determinations cannot provide an exact quantitative measure of progesterone production but reflect to some extent the output of the hormone by the corpus luteum or the placenta

Pregnanediol is excreted in the urine in the follicular as well as in the luteal phase of the normal menstrual cycle The steroid is also present in the urine of children, of men and

of post menopausal women During normal pregnancy the pregnanediol excretion gradually increases and after delivery the levels fall rapidly It is suggested that when the presence of an active corpus luteum or a placenta can be excluded, the urinary pregnanediol is derived from precursors which have been secreted by the adrenal cortex

Pregnanediol assays may sometimes be of value in the investigation of menstrual abnormalities and as an index of placental function during pregnancy Recent work has indicated that urinary pregnanediol determinations may provide a useful index of adrenocortical activity in men and in post menopausal women

Claims made some years ago that the uterus was necessary for the conversion of progesterone to pregnanediol and that progesterone metabolism was abnormal in patients with rheumatoid arthritis have not been subsequently confirmed

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# CHAPTER XI

# *Corticosteroids*

## INTRODUCTION

AS emphasised by Borth (1956) it is a difficult problem to arrive at a satisfactory definition of the term 'cortico steroid' The word could be used to include all the thirty compounds listed by Reichstein (1950, 1954) which contain the steroid nucleus and which have been identified in ox adrenal extracts Such a definition would probably be too wide in its scope as it would include substances like progesterone, œstrone and certain C 19 steroids which possess androgenic activity and which are closely related in structure to the male sex hormone testosterone Another possibility would be to restrict the definition to the physiologically active compounds—the so called cortin steroids (Fig 55)—which are capable of maintaining life in and correcting the metabolic abnormalities of adrenalectomised animals This definition would, in its turn, be too narrow as it would exclude the biologically inactive metabolites which, at least in urinary extracts account for the main part of the steroids present A suitable com promise might be to define the corticosteroids as C 21 steroids which contain three or more oxygen atoms and which are found in the adrenals, in the urine or in both urine and adrenals

### 1 The 'Cortin' Group of Adrenocortical Steroids

*In Fig 55 are shown the formulæ of the seven most important* biologically active corticosteroids which have so far been isolated from adrenocortical tissue Throughout the remainder of this chapter these seven compounds will be referred to by the following names—*cortisol, cortisone corticosterone,* 11 *dehydro corticosterone, deoxycorticosterone,* 11 *deoxycortisol* and *aldosterone*

It will be noted that all these steroids possess a $\Delta^4$ 3 *ketone group* which is believed to be essential for cortin activity In addition, all the substances have a characteristic grouping in

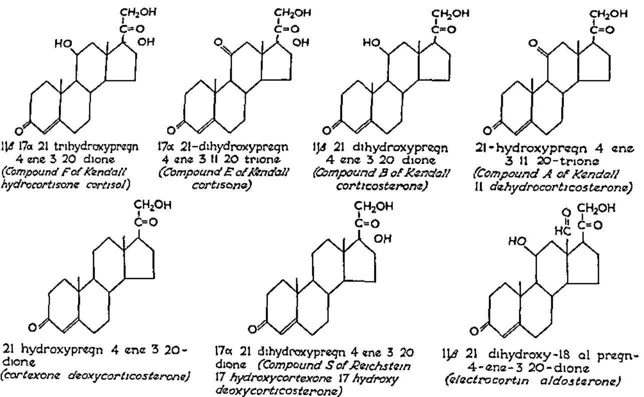



Fig 55

Structural formulæ of various corticosteroids

the side chain attached at C 17 This is the *a* ketol grouping $\left(\begin{array}{cc} CH & -C- \\ | & \| \\ OH & O \end{array}\right)$ which is important in all tests of adrenal function with the exception of life maintenance in adrenalectomised animals It also confers on the molecule strong reducing characteristics and this property has been used in certain chemical assay methods for the determination of corticosteroids in human urine The chemical structure of aldosterone is unique among the adrenal steroids in showing the presence of an aldehyde $\left(-C\begin{array}{l} \diagup H \\ \diagdown O \end{array}\right)$ group at C 18

Corticosterone, 11 dehydrocorticosterone, cortisone and cortisol have an oxygen substituent at C 11 either as a hydroxyl (-OH) or as an oxo (=O) group These substances are particularly active in relation to carbohydrate metabolism and are relatively inactive with respect to electrolyte metabolism and life maintenance in adrenalectomised animals They have been termed glucocorticosteroids On the other hand, deoxycorticosterone which lacks the oxygen substituent at C 11, is relatively weak in its action on carbohydrate metabolism but has pronounced effects on electrolyte and water metabolism and shows a high life maintaining activity This substance has been termed a mineralocorticosteroid Aldosterone is more active than deoxycorticosterone in relation to life maintenance and electrolyte metabolism and is probably the principal adrenocortical hormone concerned with the regulation of salt excretion in man It also produces effects on carbohydrate metabolism but is much weaker in this respect than either cortisone or cortisol

## 2 Steroids secreted by the Human Adrenal Cortex

The corticosteroid content of human adrenal venous blood and of human peripheral blood has been studied by numerous investigators including Morris and Williams (1953), Romanoff *et al* (1953), Bongiovanni *et al* (1954 *a*) Sweat (1955), Hudson and Lombardo (1955) Bush *et al* (1956) and Ayres *et al* (1957 *a*) Cortisol and corticosterone have been detected in adrenal venous blood while cortisol, corticosterone aldosterone, tetrahydrocortisone and possibly cortisone have been detected in peripheral blood Probably the most detailed analysis yet conducted is that of Hudson and Lombardo (1955) who

studied the corticosteroid content of adrenal venous blood in a patient suffering from mammary carcinoma before and after the administration of ACTH

There is general agreement that cortisol and corticosterone are the two main adrenocortical hormones present in human blood and that cortisol is secreted in much larger quantities than is corticosterone The evidence available at present indicates that in man, cortisol is the principal corticosteroid concerned with the regulation of carbohydrate metabolism, while aldosterone is the principal steroid concerned with the regulation of salt excretion

It is widely believed that œstrogens and progesterone are secreted by the human adrenal cortex The evidence for this view is mainly indirect and has already been discussed in Chapters IX and X

It is well established that the 17 ketosteroids in urine originate to some extent from precursors secreted by the adrenals The compounds probably arise for the most part from the metabolic breakdown in tissues other than the adrenals of certain of the C 21 steroids Recently, however, Romanoff *et al* (1953), using adrenal venous blood have detected the presence of two C 19 steroids bearing a close structural relationship to testosterone This finding suggests that the urinary 17 ketosteroids may also originate from C 19 steroids which are secreted as such by the adrenal glands

Romanoff *et al* (1953) and others have been able to detect in human adrenal venous blood physiologically inactive steroids structurally related to cortisone and cortisol Accordingly it can be concluded that such steroids are not only synthesised in the adrenals but are also secreted by the glands themselves

It should not be assumed that all of the steroids present in the adrenal glands of man and animals are necessarily secreted into the blood Furthermore it should not be concluded that human adrenal glands necessarily synthesise the same steroids as are found in the glands of other species

## THE ESTIMATION OF CORTICOSTEROIDS BY BIOLOGICAL METHODS

In studies on patients bio assay methods have been applied mainly to urine extracts and few investigations have been

conducted on blood Biological tests determine cortin' activity in urine They are generally carried out on adrenal ectomised rodents and are based on the ability of the physiologically active corticosteroids to produce certain effects in these animals such as prolongation of life, protection against cold glycogen deposition in the liver, regulation of electrolyte balance and eosinopenia For further information regarding the very large number of methods available for the biological assay of the corticosteroids the reader is referred to review articles by Vogt (1948), Sayers (1950), Loraine (1952), Dorfman (1953) and Diczfalusy *et al* (1956)

Bio-assay procedures provide information regarding the urinary excretion of the physiologically active corticosteroids such as cortisone cortisol and aldosterone However they do not measure any of the physiologically inactive metabolites of these hormones which in relation to the hormones themselves, form much the larger part of the steroids excreted Accordingly although biological methods of assay may yield valuable information of a qualitative nature regarding the response of the adrenals to stimulation, they are obviously unsatisfactory for the quantitative assessment of adrenocortical secretory activity In addition, such methods are usually expensive, laborious and tedious and are quite unsuitable for routine use in clinical studies

Of the many methods of bio assay which have been proposed the following only will be considered

1 The test depending on *glycogen deposition in the liver* of adrenalectomised mice (Venning *et al*, 1946, Eggleston *et al*, 1946 Dorfman *et al*, 1946)
2 The *cold protection* test in adrenalectomised rats (Selye and Schenker, 1938)
3 The test depending on the production of *eosinopenia* in adrenalectomised mice (Speirs and Meyer 1951)
4 Tests depending on *electrolyte excretion* in adrenalectomised rats (Dorfman 1950, Simpson and Tait, 1952)

## 1 Glycogen Deposition Test

This method depends on the ability of urine extracts with 'cortin like' activity to promote deposition of glycogen in the liver of adrenalectomised rodents Tests involving mice are

some ten to twenty times more sensitive than those employing rats and are therefore to be preferred for use in the clinical field The method is specific for corticosteroids with an oxygen atom at C 11, results of assays are generally expressed in terms of cortisone as ' cortisone equivalents In the hands of Venning *et al* (1946) the test was reasonably precise, the index of precision (λ) being generally below 0 2

Several workers including Venning *et al* (1946) and Forbes *et al* (1950) have studied the excretion of glycogenic cortico steroids in normal and pathological conditions in man Their results which are now mainly of historical interest, will be discussed briefly later in this chapter

### 2 Cold Protection Test

This technique is based on the ability of urinary extracts to prolong the survival time of adrenalectomised rats on exposure to low environmental temperature The reliability criteria of this method have been reviewed by Vogt (1948) The test is more sensitive but less specific than that involving glycogen deposition in the liver it has, however a relatively low degree of precision and shows dose response curves which are very variable in slope For these reasons the method has not been widely used in clinical studies

### 3 Mouse Eosinophil Test

This method was originally described by Speirs and Meyer (1951) and depends on the ability of adrenal steroids with an oxygen atom at C 11 to cause a fall in the circulating eosinophils in adrenalectomised mice which have been pre treated with adrenaline The test is very sensitive and will detect as little as 1 $\mu$g of cortisone and cortisol It is also reasonably specific for steroids with an oxygen atom at C 11

The reliability criteria of the mouse eosinophil test have been intensively studied by Bibile (1953) This worker also found it to be both sensitive and specific and showed that the slopes of the dose response curves remained relatively constant throughout the year It was, however, demonstrated that the method had a very low degree of precision the index of precision (λ) was generally greater than 0 5 and a fourfold increase in dosage was required in order to obtain a significant

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difference between the mean responses of two groups each of ten mice

*In view of the large error of this test it is unlikely that it will provide results of any quantitative significance when applied to clinical problems*

## 4 Electrolyte Excretion Tests

Although methods have existed for some time for the measurement of so-called glucocorticosteroid activity in body fluids it is only within recent years that bio assays have been developed for the quantitative determination of ' mineralo corticosteroid activity in blood and urine extracts The index of mineral activity may be based on the estimation of either inert or of radio active elements Deming and Luetscher (1950), Spencer (1950) and others described assay methods in adrenalectomised rats in which the quantitative determination of mineral activity was based on the retention of inert sodium while Dorfman and his co workers (Dorfman *et al* 1947, Dorfman 1950) also using adrenalectomised rats described two separate bio assay methods, one depending on the retention of radio active sodium and the other on the excretion of radio-active potassium

In 1952 Simpson and Tait developed a bio assay method for mineralocorticosteroid activity which is probably superior to any that have hitherto been proposed This procedure (the mineral ratio test) is based on the ability of corticosteroids to depress the urinary $^{24}Na/^{42}K$ ratio in adrenalectomised rats in a two hour period after the injection of the radio active isotopes In Simpson and Tait s original experiments results were expressed in terms of deoxycorticosterone acetate (DCA) Over the dosage range employed the depression of the ratio was found to bear a linear relationship to the dose of DCA administered The mineral ratio test was shown to be reasonably specific the 11 oxygenated steroids viz, cortisone cortisol and corticosterone were relatively inactive by this technique while other steroids such as œstradiol 17β progesterone and testosterone were completely inactive The error of the test was not unduly large the index of precision (λ) generally lay between 0 2 and 0 3

This bio assay method proved very helpful in the series of experiments which culminated in the isolation of *aldosterone*

from adrenal extracts (Simpson *et al*, 1953, 1954 *a b, c*) Using this technique, Speirs *et al* (1954) demonstrated that aldosterone was approximately 120 times more active than deoxycorticosterone

## THE ESTIMATION OF CORTICOSTEROIDS IN URINE BY CHEMICAL METHODS

Many types of steroid of adrenocortical origin can be determined in urine by chemical means Perhaps the most important of these from the clinical point of view are the *neutral urinary 17 ketosteroids* although it must be borne in mind that, in the male approximately one third of this fraction is accounted for by metabolites of testosterone, the hormone secreted by the testes The methods of estimation of these substances together with the clinical applications of urinary 17 ketosteroid assays, are discussed in detail in Chapter XII

Many of the chemical procedures which have been used to determine the urinary excretion of corticosteroids in man are far from satisfactory from the quantitative point of view Some of the deficiencies in existing methods have been reviewed by Marrian (1951, 1955) It is for example well known that present techniques of hydrolysis and extraction of the urinary corticosteroids are by no means optimal and that for this reason many of the procedures in current use estimate only a small proportion of the total corticosteroids in a given urine sample Furthermore some of the chemical reactions employed are far from specific for the urinary adrenocortical steroids in that many unrelated substances both steroidal and non steroidal may be estimated This latter criticism is particularly true in the case of techniques depending on the reducing properties of urinary adrenocortical hormones and on their ability to generate formaldehyde on oxidation with periodic acid

It can be confidently predicted that, with the passage of time chemical methods will entirely replace bio-assay techniques for the quantitative determination of urinary adrenocortical steroids In recent years great advances have been made in the sphere of corticosteroid methodology and it is reasonable to expect that in the not too distant future chemical assay methods satisfactory in terms of accuracy precision sensitivity and specificity will be developed One of the great achievements

in the last decade has been the introduction of paper chromatographic methods for the separation and identification of the individual steroids of adrenocortical origin in urine extracts Another important development has been a better understanding of hydrolysis conditions for urinary corticosteroids and the introduction of hydrolysis by enzymes such as β glucuronidase in place of hydrolysis by acid This modification has been shown to cause a great increase in the yield of corticosteroids from urine extracts As more reliable chemical methods are developed for the assay of urinary adrenocortical steroids, it is probable that estimations of these hormones will become of increasing diagnostic and prognostic value in relation to clinical problems in man

The great majority of chemical methods for the estimation of corticosteroids are based on different colour reactions which are produced by functional groups in the corticosteroid molecule Table XVII, which is taken from a recent article by Diczfalusy *et al* (1956) shows some of the colour reactions which have been employed by various investigators

Prior to estimation by colorimetry the corticosteroids must first be extracted from urine and fractionated The difficulties involved in these latter two procedures will be discussed under the individual methods

The following chemical assay methods will be considered

1 Methods for reducing steroids
2 Methods for formaldehydogenic steroids
3 Methods for acetaldehydogenic steroids
4 Methods developed by Norymberski *et al*
5 Methods for 17 21 dihydroxy 20 keto corticosteroids (Porter Silber chromogens)
6 Paper chromatographic methods
7 Methods for aldosterone

Mention will also be made in a separate section of problems associated with the hydrolysis of conjugated C-21 urinary adrenocortical steroids

## 1 Methods for reducing Steroids

These depend on the fact that C 21 urinary adrenocortical steroids possess an α ketol side chain which has marked reducing properties (see Table XVII) In 1945 Talbot *et al* described a method for determining reducing steroids in purified chloroform

SOME CHEMICAL ASSAY METHODS FOR CORTICOSTEROIDS
(*After D c f lusy et al* 1956)

| METHOD OF ESTIMATION | REACTING GROUP | | | | | | | REFERENCE |
|---|---|---|---|---|---|---|---|---|
| | αβ- t d 3 k t | CH C=O OH 17 20 k l | CH CHOH OH 17 20-gly l | CH OH CHOH 20 21 gly l | CH OH C=O 20 21 k t l | CH OH CHOH OH 17 20 21 l | CH OH C=O OH 17 21-d l 20-o (d hyd o y de h ) | |
| R d t f pp fcts | — | — | — | — | + | — | + | Talbo t l (1945) |
| R d t f ph ph m lybd acd | + | — | — | — | + | — | + | H d & Sob l (1946) |
| R d ct of t t az l m | — | — | — | — | + | — | + | Ch & T w ll (1951) |
| O d t t f m ld hyd by p d d | — | — | — | + | + | + | + | Low t t l (1946) |
| O d i t ld hyd by p d c d | — | — | + | — | — | — | — | Co (1952) |
| R ct w th ph ylhyd azi d lph t d (P S lb r re t ) | — | — | — | — | — | — | + | Port & S ib (1950) |
| R t o w h d t ph ylhydra i ei lk l l t | + | + | — | — | + | — | + | G all & M Do ld (1953) |
| O d t 17 k t t d by p d a d | — | — | + | — | — | + | — | Talb t & E u go (1944) |
| O d t o 17 k ds by h m d | — | + | + | — | — | + | + | W l & F b ks (1955) |
| O id ti to 17 k t t d by d m b m th t | — | — | + | — | — | + | + | N rymb k (1952) |
| O d t 17 k t d by sod m b m h t ft e- d cti by d m b hydr d | — | + | + | — | — | + | + | Appl by t l (1955) |
| O d ti by d m b m h t ad ct by d m bo o- hyd ide, d by d m b m tha e | — | + | — | — | — | — | — | Appl by t l (1955) |
| Abso p n of t d t 240 mμ f th s m baz e 302 mμ | + | — | — | — | — | — | — | Zaff (1953) |

extracts of urine by means of an alkaline copper reagent, while in the following year Heard and Sobel (1946) described a somewhat similar method in which phosphomolybdic acid was used for the determination of reducing power

Methods for estimating reducing steroids in urine were of some value in the past as an index of adrenocortical function in health and disease However, it is now clearly recognised that such techniques are far from specific in that many substances in urine other than the a ketolic steroids affect the results obtained In view of the development in more recent years of more reliable methods for the quantitative determination of corticosteroids in human urine, techniques depending on the reducing properties of C 21 steroids should no longer be employed in clinical practice

## 2 Methods for Formaldehydogenic Steroids

This technique was first described by Lowenstein *et al* (1946) and was subsequently modified by other workers including Daughaday *et al* (1948 *a*), Corcoran and Page (1948) and Hollander *et al* (1951) It has been widely used in clinical studies The chemical principle involved in this method depends on the fact that any structure in which there is a primary alcoholic ( $-CH_2OH$) group adjacent to either a ketonic ($C=O$) group or another alcoholic group will yield formaldehyde on oxidation with periodic acid Since the C 21 adrenocortical hormones and many of their metabolic reduction products have an α ketol $\left(\begin{array}{c} CH_2OH \\ | \\ C=O \\ | \end{array}\right)$ or α glycol $\left(\begin{array}{c} CH_2OH \\ | \\ CHOH \\ | \end{array}\right)$ side chain, these substances, on oxidation with periodic acid, will yield formaldehyde which can be determined colorimetrically by means of chromotropic acid

While this method is probably somewhat more specific than that depending on the reducing properties of α ketolic steroids, it has been definitely shown that urine extracts contain substances other than steroids which yield formaldehyde on oxidation with periodic acid Another source of error arises from the fact that solvents such as chloroform and ether, used for the extraction of the urine may contain small amounts of formaldehydogenic impurities which are very difficult to remove completely by repeated distillation

Marrian (1951) has emphasised the limitations, from the quantitative point of view of methods based on formaldehydogenic steroids It is probable that such techniques measure only a small proportion of the total corticosteroids present in urine In view of the development of more reliable methods for the assessment of adrenocortical function, *e g* techniques depending on the estimation in urine of 17 ketogenic steroids it is unlikely that in the future formaldehydogenic procedures will continue to be used in clinical investigations

### 3 Methods for Acetaldehydogenic Steroids

Cox (1952) developed a quantitative method for urinary adrenocortical steroids possessing a 17 20 dihydroxy 20 methyl side chain $\left(CH_3—\underset{OH}{CH}—\underset{OH}{C}—\right)$ He showed that steroids with such a side chain yielded acetaldehyde on oxidation with periodic acid by cleavage of the side chain between carbon atoms 17 and 20 It was also found that the acetaldehyde liberated could be quantitatively determined by means of a colour reaction with 4 hydroxy diphenyl and that the procedure could be applied to the estimation of acetaldehydogenic steroids in urine extracts

It must be remembered that this technique measures only one particular group of urinary corticosteroids and therefore by itself it does not provide an adequate means of assessing adrenocortical activity However the method may be of limited value for this purpose if combined with methods specific for other types of steroids

One of the most important steroids determined by Cox s method is the compound 5$\beta$ pregnane 3$\alpha$ 17$\alpha$ 20$\alpha$ triol (pregnanetriol) According to Bongiovanni and his co workers (Bongiovanni 1953, Bongiovanni *et al* 1954 *b*) this substance is excreted in relatively large quantities by patients suffering from the adrenogenital syndrome

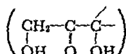
### 4 Methods developed by Norymberski *et al*

These fall into three main groups

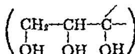
(*a*) Method for 17 ketogenic steroids
(*b*) Method for total 17 hydroxysteroids
(*c*) Method for ' 21 deoxyketols

(*a*) METHOD FOR 17-KETOGENIC STEROIDS.—Norymberski and his co-workers (Norymberski, 1952, Norymberski *et al*, 1953) have described a method for determining a structurally well-defined group of urinary adrenocortical steroids which they termed the 17-*ketogenic steroids*. The chemical principle involved in this method depends on the fact that C-21 adrenocortical hormones with certain characteristic side chains can be smoothly oxidised by means of sodium bismuthate to 17-ketosteroids. As shown in Table XVII, the three groups of compounds giving this reaction are as follows:

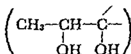
(i) 17, 21-dihydroxy-20-keto or 'dihydroxyacetone' steroids



(ii) 17, 20, 21-trihydroxysteroids



(iii) 17, 20-dihydroxy-21-deoxysteroids



It should be noted that steroids with the 17-hydroxy-20-keto-21-deoxy side chain ($CH_3$—C(=O)—C(OH)—) are not so oxidised.

The oxidation with sodium bismuthate can readily be performed on human urine. If it is followed by acid hydrolysis the 17-ketosteroids originally present in urine along with those formed by the bismuthate oxidation can be extracted and estimated by the usual Callow-Zimmermann procedure. If an estimation of the 17-ketosteroids originally present is undertaken on a separate aliquot of the same urine specimen, the difference between the two results will give a value for the 17-ketosteroids formed by bismuthate oxidation from the above-mentioned three groups of C-21 17-hydroxysteroids. Norymberski *et al*

(*c*) METHOD FOR '21 DEOXYKETOLS'.—This technique, which measures selectively a group of compounds with the 17 hydroxy 20 keto 21 deoxy side chain $\left( CH_3{-}\underset{\underset{O}{\|}}{C}{-}\underset{\underset{OH}{|}}{\overset{/}{C}}{-} \right)$ was described by Appleby and Norymberski in 1954. The urinary 21 deoxyketols are converted into 17 ketosteroids by consecutive treatment with sodium bismuthate, sodium borohydride and sodium bismuthate. The final colorimetric determination depends on the Zimmermann reaction. During the procedure all the other ketonic and ketogenic compounds present in the analysed mixture are converted into alcohols and do not interfere with the final result. One of the more important steroids possessing this side chain is 17α hydroxypregnanolone, a compound originally isolated from urine by Dobriner *et al* (1951) and believed by these workers to be characteristic of patients with rheumatoid arthritis.

Appleby and Norymberski (1955) have studied the excretion of 21 deoxyketols in normal and pathological conditions in man. Their results will be discussed later in this chapter.

GENERAL CONCLUSIONS REGARDING THE NORYMBERSKI METHODS.—At the time of writing the evidence indicates that the Norymberski methods, although not yet completely satisfactory from the quantitative point of view, are probably the most reliable techniques available for the determination of corticosteroids in urine. Accordingly, if an assessment of adrenocortical function is to be made by urinary assays, the Norymberski techniques should be used in preference to such methods as those depending on the reducing or formaldehydogenic properties of adrenocortical steroids.

In the opinion of Diczfalusy *et al* (1955) the method for urinary 17 ketogenic steroids developed by Norymberski *et al* (1953) has the following advantages: (i) The technique circumvents the difficulties associated with the hydrolysis of C 21 17 hydroxycorticosteroids. (ii) Normal excretion values are higher than those reported in the case of previously published methods. This is important in view of the fact that the reliability criteria of the technique are probably more satisfactory than those of previous methods. (iii) The procedure measures predominantly steroidal material and the components

identified following chromatography are the expected derivatives of known urinary 17 ketogenic steroids (iv) The method is simple and rapid and is well suited for routine use in a clinical laboratory

## 5 Methods for 17 21 dihydroxy 20 keto-corticosteroids[1]

This group consists of adrenocortical steroids possessing the dihydroxyacetone side chain $\left(\begin{array}{ccccc} CH_2 & - & C & - & \overset{/}{C}- \\ | & & \| & & | \\ OH & & O & & OH \end{array}\right)$ and includes such compounds as cortisone, cortisol and tetrahydrocortisone In 1950 Porter and Silber described a colour reaction with phenylhydrazine and sulphuric acid which is specific for compounds of this type and in 1952 Nelson and Samuels made successful use of this reaction in a method for the determination of these hormones in blood (see p 264) Since then various attempts have been made to adapt the Porter Silber reaction to the estimation of steroids in urine extracts In the method described by Reddy and his co workers (Reddy *et al* 1952, Reddy 1954) the urine is acidified to *p*H 1 and is saturated with sodium sulphate, the corticosteroids are then extracted with butanol and the butanol extract is treated with sodium carbonate before being subjected to colorimetry by the Porter Silber reaction In the technique of Glenn and Nelson (1953) the corticosteroid conjugates are hydrolysed by $\beta$ glucuronidase and are extracted with chloroform the free steroids are then chromatographed on magnesium trisilicate columns prior to colorimetric estimation

It must be emphasised that neither of these methods is completely satisfactory from the quantitative point of view In the technique of Glenn and Nelson (1953) the conditions of hydrolysis are probably not optimal (see p 261) and for

[1] For estimations in which the final colorimetric determination depends on the Porter Silber reaction, this term should be used in preference to 17 hydroxycorticosteroid The latter has a relatively wide connotation and includes compounds with the 17 20 ketol 17 20 glycol and 17 20 21 triol as well as dihydroxyacetone side chain As will be seen in Table XVII only the last mentioned group reacts with phenylhydrazine and sulphuric acid In the opinion of many investigators the material giving the Porter Silber reaction is best referred to as Porter Silber chromogen. The use of this term would appear to be justified in view of the fact that in the relatively impure extracts obtained from blood and urine substances other than steroids are capable of affecting the reaction

(c) METHOD FOR 21 DEOXYKETOLS'—This technique which measures selectively a group of compounds with the 17 hydroxy 20 keto 21 deoxy side chain $\left(CH_3-\underset{\Vert}{\overset{}{C}}-\underset{|}{\overset{/}{C}}-\right)$ was
O OH
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### 7 Methods for Aldosterone

Within recent months chemical assay methods for urinary aldosterone have been described by a number of investigators, including Neher and Wettstein (1955 1956), Ayres *et al* (1956 1957 *b*) and Moolenaar (1956) At the time of writing, little information is available regarding the reliability of these procedures, and none of them have been used to any extent in clinical studies

In the technique of Ayres *et al* (1956, 1957 *b*) the urinary excretion of cortisol and of corticosterone as well as of aldo sterone can be determined The main steps in this method are as follows (*a*) Acidification of the urine to *p*H 1 (*b*) continuous chloroform extraction, (*c*) chromatography on silica, (*d*) chromatography on celite (*e*) acetylation, (*f*) chromatography on paper (*g*) final estimation of the individual compounds by fluorimetry

When aldosterone, cortisol and corticosterone were added to urine and extracted as described above the mean recovery figures for all three compounds ranged from 70 to 80 per cent The method has a relatively high degree of sensitivity and will measure with reasonable precision as little as 2 to 3 $\mu$g of aldosterone The chief disadvantage of the procedure lies in the fact that it is very exacting and very laborious, the method in its present form is certainly not suitable for routine use in the clinical field although it may give useful information in selected cases

## Hydrolysis of Conjugated C-21 Urinary Corticosteroids

This subject has been recently reviewed by Marrian (1955) and the present account is based on his conclusions

Corticosteroids are excreted in urine partly in the free unconjugated state but mainly conjugated with glucuronic acid as glucuronides It is also probable that small proportions of the urinary corticosteroids are conjugated with sulphuric acid and excreted as sulphates As in the case of œstrogens and pregnanediol it is first necessary to hydrolyse the con jugates in order to obtain the free steroid which can then be extracted by a suitable solvent *e g* chloroform The traditional method of hydrolysis consists of heating the urine with strong mineral acid This technique has been used with some success

this reason significant losses of corticosteroids occur in the course of the method The main difficulty in the technique of Reddy *et al* (1952) arises from the high background absorption found in the final colorimetric determination Such high 'blank' values occur because the urine extracts are insufficiently purified prior to colorimetry Although the Porter Silber reaction will yield quantitatively reliable results with urine extracts which have been carefully purified by chromatographic means, the test cannot be expected to be so satisfactory when it is applied to the relatively crude material obtained in this procedure Hertoghe *et al* (1955) have claimed that the reliability of Reddy's method can be somewhat increased by using the correction formula described by Allen (1950) in the final colorimetric determination In this formula the optical density is obtained by making readings at three wave lengths The correction is valid provided that the three optical densities of the non specific chromogenic material lie on a straight line (see p 164)

## 6 Paper Chromatographic Methods

These were originally developed by Zaffaroni and his co workers in the United States (Zaffaroni *et al* 1950, Zaffaroni and Burton 1951, Zaffaroni 1953) and by Bush in the United Kingdom (Bush, 1952, 1953) Such methods have made it possible for the first time to determine the individual adrenocortical steroids in urine extracts Many solvent systems are now available which allow a satisfactory resolution of steroid mixtures on paper strips and a variety of different tests have been described which allow of the location and identification of spots due to the individual steroids For example steroids with a $\Delta^4$ 3 ketone grouping such as progesterone can be identified on paper by their characteristic u v absorption, 17 ketosteroids by the use of the Zimmermann reaction and substances with a reducing $\alpha$ ketolic side chain by means of certain tetrazolium compounds which become reduced to give pink or blue colours

As stated above the chief value of paper chromatographic methods when applied to urine extracts lies in the identification of individual steroids Such techniques at present are not strictly quantitative although it may in the future, be possible to adapt them for quantitative work

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to hydrolyse the conjugated forms of 17 ketosteroids œstrogens and pregnanediol, but cannot be employed in the case of C-21 adrenocortical steroids, since the compounds in this group which are most important from the quantitative point of view are highly unstable in hot acid due to the presence of a tertiary hydroxyl group at C 17 Some investigators in estimating reducing and formaldehydogenic steroids, attempted to overcome this difficulty by using acid hydrolysis at room temperature This procedure is also highly unsatisfactory for although it may hydrolyse some of the steroid sulphates, it is unlikely that it will cause any significant hydrolysis of urinary glucuronides Indeed, it is probable (Marrian 1951) that workers using this technique were only able to determine approximately 5 to 10 per cent of the reducing or formaldehydogenic material present in urine extracts

Another and definitely superior method for the hydrolysis of urinary glucuronides depends on the incubation of the urine with the enzyme *β glucuronidase*, active preparations of which can be obtained from liver and spleen and from certain bacteria and molluscs This form of enzymic hydrolysis gives a much higher yield of free C 21 urinary adrenocortical steroids than is obtained by acid hydrolysis, and the procedure is now widely used as an early step in the determination of formaldehydogenic and 17 21 dihydroxy 20 ketosteroids in urine and in the estimation of the individual corticosteroids by paper chromatographic techniques Although the routine use of β glucuronidase hydrolysis is probably justifiable on the grounds that it is the best method of hydrolysis at present available, it should nevertheless be borne in mind that the procedure may not be entirely trustworthy in quantitative work Its chief disadvantages arise firstly from the presence of various glucuronidase inhibitors in urine and secondly from the fact that hydrolysis of urinary sulphates does not occur

Little information is available on the use of the enzyme *sulphatase* for the hydrolysis of urinary steroid conjugates However it is already clear from the work of Savard *et al* (1954) and of Roy (1956) that sulphatases from different sources are highly selective in their action Accordingly it is unlikely that one particular sulphatase preparation would be suitable for the hydrolysis of all steroid sulphates in urine

## THE ESTIMATION OF CORTICOSTEROIDS IN BLOOD BY CHEMICAL METHODS

At the present time chemical techniques for the determination of corticosteroids in blood have certain limitations from the quantitative point of view and further methodological work is necessary before such procedures can be used with confidence in routine clinical studies One of the chief difficulties arises from the fact that little is known regarding the relative proportion of free and conjugated corticosteroids in human blood In earlier assay methods such as that of Nelson and Samuels (1952) it was assumed that corticosteroids in plasma occur mainly in the free state and that, for this reason, it is unnecessary to incorporate a hydrolysis step in the procedure However more recent work by Bongiovanni and his co workers (Bongiovanni 1954, Bongiovanni *et al* 1954 *a*) in which hydrolysis with the enzyme β glucuronidase was employed has demonstrated that approximately half of the corticosteroids in plasma are present in the conjugated rather than the free form In 1955 Weichselbaum and Margraf developed a method for the separate determination of the free and conjugated adrenocortical steroids in plasma This technique although somewhat laborious is probably more reliable from the quantitative point of view than any previously proposed Its application to clinical problems will be awaited with interest

In most of the techniques in current use extracts containing the corticosteroids are purified by chromatography either on columns or on paper Procedures employing column chromatography have been described by various investigators including Nelson and Samuels (1952) Morris and Williams (1953) and Weichselbaum and Margraf (1955) while separation techniques depending on paper chromatography have been devised by Simpson *et al* (1952) Bush and Sandberg (1953) and others The method which has been most widely employed in the clinical field is that of Nelson and Samuels (1952) and this will be discussed in more detail below

It can be confidently predicted that with the passage of time paper chromatographic methods will come into general use for the estimation of corticosteroids in blood as well as in urine By use of such techniques it has already been possible

to show that the main adrenocortical steroids in human adrenal venous blood are cortisol and corticosterone and that the same two steroids are also present in readily detectable amounts in human peripheral blood (see p 246) Further work along these lines should greatly increase our knowledge of adreno cortical function in health and disease and should provide valuable information in relation to the therapy of adrenocortical disorders in man

## The Method of Nelson and Samuels (1952)

The main steps in this procedure are (1) extraction of blood or plasma by an ether chloroform mixture, (2) partition of the ether chloroform soluble material between ethanol and hexane (3) chromatography of the ethanol soluble fraction on Florosil columns, (4) colorimetric determination of the 17 21 dihydroxy 20 keto corticosteroids by a micro modification of the Porter Silber reaction (see p 259)

This method has been used in several laboratories, particularly in the United States and in the hands of some investigators it has yielded information of clinical importance regarding the secretory activity of the adrenal cortex in health and disease However, equal success in reproducing the method was not attained in all laboratories, and various modifications of the original technique were proposed (Weichselbaum *et al*, 1953 Bayliss and Steinbeck 1953) In 1953 Eik Nes, Nelson and Samuels acknowledged that difficulties had been encountered by others in reproducing the method, they published additional technical details and emphasised the importance of careful preparation of materials, particularly the chromatographic adsorbent Florosil

Recently Harwood and Mason (1956) have made a careful and detailed study of the reliability criteria of the Nelson Samuels method They concluded that the technique although difficult to perform, was reasonably satisfactory for the determination of 17 21 dihydroxy 20 keto corticosteroids in human plasma

It must be borne in mind that the Porter Silber reaction, when applied to the relatively impure extracts of blood and plasma, almost certainly measures non specific chromogenic material in addition to 17 21 dihydroxy 20 keto cortico steroids (see p 259) For this reason it is probably more

correct to speak of the final material estimated as Porter Silber chromogens' rather than to refer to it as a specific group of steroid hormones

## THE CHOICE OF A CORTICOSTEROID ASSAY METHOD FOR CLINICAL PURPOSES

In the case of the œstrogens pregnanediol and the 17 ketosteroids it was possible to make definite recommendations as to what method or group of methods should be used in clinical practice (see Chaps IX X and XII) It is much more difficult to make similar recommendations in the corticosteroid field because, at the present time methods of assay of adrenocortical hormones and of their metabolites are les satisfactory from the quantitative point of view than are the best methods for the other steroids mentioned above Nevertheless, an attempt will be made to draw a few tentative conclusions in the hope that these will be helpful to workers desirous of using a corticosteroid assay method in clinical investigations

1 Bio assay methods such as those depending on eosinopenia in mice or on glycogen deposition in the liver of rodents should no longer be employed These techniques are expensive and laborious and do not yield results of much quantitative significance

2 Chemical assay methods depending on the reducing and formaldehydogenic properties of adrenocortical steroids show a low degree of specificity and should now be replaced by more reliable procedures

3 Relatively crude chemical assay methods such as that developed by Reddy *et al* (1952) may be of value in assessing whether or not the corticosteroid excretion in urine rises following *the administration of adrenocortical hormones or of* ACTH but are of little value in quantitative studies

4 One or other of the methods described by Norymberski and his co workers is probably the most reliable procedure at present available for the estimation of corticosteroids in urine

5 Chemical assay methods for aldosterone will probably soon replace bio assay techniques The method recently developed by Ayres *et al* (1956 1957 *b*) appears promising for use in selected cases but is too laborious for routine use

6 In the future assay methods for corticosteroids in blood and urine will probably depend on paper chromatographic techniques or on procedures involving radio-active steroids

## SOME GENERAL PRINCIPLES IN THE CLINICAL APPLICATION OF CORTICOSTEROID ASSAYS

This subject has recently been reviewed by Diczfalusy *et al* (1956) and the present account is based mainly on the conclusions of these workers

In the corticosteroid field it is convenient to divide disease states into two groups

1 Conditions with an increased corticosteroid excretion.
2 Conditions with a decreased corticosteroid excretion

### 1 Conditions with an increased Corticosteroid Excretion

In Cushing s syndrome resulting from carcinoma of the adrenals a marked increase in corticosteroid excretion occurs Abnormally high values are also found in patients with adrenal hyperplasia and with benign tumours of the adrenal cortex The excretion of corticosteroids is generally raised in cases of congenital adrenal hyperplasia (see p 301) and in conditions of stress In patients with normal adrenal function the parenteral administration of ACTH causes a marked increase in corticosteroid levels over pre treatment values

It is of interest that in certain disease states the urinary excretion of C-21 corticosteroids does not parallel that of the urinary 17 ketosteroids For example, in cases of benign tumour of the adrenal cortex the corticosteroid excretion is generally abnormally high while the 17 ketosteroid output may be within or only slightly above the normal range On the other hand, in patients with testicular and ovarian tumours producing symptoms of virilism the high excretion of 17 ketosteroids is usually associated with normal corticosteroid levels in urine

### 2 Conditions with a decreased Corticosteroid Excretion

In Addison s disease very low corticosteroid values in urine are generally found and in a fully developed case not receiving therapy zero values have been reported In Addison s disease

in men urinary corticosteroid determinations may be more informative than 17 ketosteroid assays as the testes contribute significantly to the latter group of steroid hormones

In Simmonds disease very low urinary levels of cortico steroids are found, while in patients with anorexia nervosa the excretion of these substances may be within the normal range In the opinion of Diczfalusy *et al* (1956) corticosteroid estimations in blood and urine following the parenteral administration of ACTH may be helpful in differentiating primary adrenal failure from adrenal failure secondary to lesions of the pituitary In Simmonds disease stimulation of the adrenals by ACTH generally causes a rise in the cortico steroid levels in blood and urine, in Addison s disease on the other hand such an increase can usually not be demonstrated Liddle *et al* (1954) believe that the urinary estimation of 17 21 dihydroxy 20 keto corticosteroids after ACTH adminis tration forms the best test of adrenocortical function at present available

## THE ESTIMATION OF VARIOUS GROUPS OF CORTICOSTEROIDS IN CLINICAL CONDITIONS

In the majority of studies in the clinical field estimations of the various groups of corticosteroids have been conducted on urine rather than on blood Recently however attempts have been made to develop quantitative methods which could be applied to blood and it is reasonable to expect that in the future parallel assays involving blood and urine will become possible Studies of this type will undoubtedly yield information of clinical importance in the investigation of adrenal disorders in man

The following groups of adrenocortical steroids will be considered

1 Glycogenic corticosteroids
2 Reducing steroids
3 Formaldehydogenic steroids
4 Steroids determined by the methods of Norymberski *et al*
5 17 21 dihydroxy 20 keto corticosteroids (Porter Silber chromogens)
6 Aldosterone

It has already been emphasised that assay methods which determine the first three groups of steroids are very

unsatisfactory from the quantitative point of view The results reported under these sections are now mainly of historic interest

## 1 Glycogenic Corticosteroids

Venning and her collaborators (Venning, 1946, Venning and Kazmin, 1946) have made extensive studies on the excretion of glycogenic corticosteroids in normal and pathological conditions Results were expressed in glycogenic units per twenty four hours, one ' unit being equivalent to the biological activity contained in 1 $\mu g$ of cortisone

(*a*) NORMAL SUBJECTS —Venning and Kazmin (1946) found that the excretion of glycogenic corticosteroids tended to be higher in healthy men than in healthy women of a comparable age group Venning *et al* (1949) showed that small quantities of glycogenic corticosteroids were excreted in the urine of newborn babies The output gradually increased with the age of the infant

It has been claimed (Venning, 1946) that the corticosteroid excretion shows a characteristic pattern during normal pregnancy In the first trimester the levels are generally above those encountered in normal non pregnant individuals, and by about the 120th day of gestation the values are again within the normal range Subsequently another rise occurs in the third trimester, at which time the excretion of glycogenic corticosteroids is much higher than at any other time during pregnancy A slight fall is noted prior to parturition and early in the puerperium the levels are again within the normal range

(*b*) *DISEASE STATES —Most investigators have found* abnormally low values in patients with Addison s disease and with panhypopituitarism In anorexia nervosa on the other hand, the levels are usually normal Cases of Cushing s syndrome and of the adrenogenital syndrome resulting either from adrenal tumours or hyperplasia, excrete very large quantities of glycogenic corticosteroids in their urine, while in patients with simple hirsutism the readings are not abnormally high

## 2 Reducing Steroids

Talbot *et al* (1951) have reviewed their wide experience with the copper reduction method in normal and abnormal

subjects An abnormally low output of reducing steroids was noted in most cases of Addison s disease, in panhypopituitarism and in myxœdema while in Cushing s syndrome, whether due to adrenal tumour or adrenal hyperplasia the excretion was generally above the normal range There was considerable overlap between the levels found in patients with cortical tumours and those obtained in cases of hyperplasia accordingly, it was concluded that the estimation of reducing steroids was not a satisfactory method of differentiating between the two conditions Assays conducted in patients with a number of miscellaneous diseases, *e g* essential hypertension rheumatic fever, simple hirsutism eunuchoidism and Turner s syndrome all gave results which lay within the normal range On the basis of some 1,400 urinary corticosteroid determinations Talbot *et al* (1951) concluded that the copper reduction method could be considered a reasonably reliable index of adrenocortical hormone production

Sprechler (1951) has made an extensive investigation of the excretion of reducing corticosteroids in normal individuals The method employed was slightly modified from that described by Heard and Sobel (1946) in which phosphor molybdic acid is used for the determination of reducing power It was found that healthy men excreted relatively more reducing steroids than healthy women of a comparable age group and that the output was lowest of all in children There was a wide variation in excretion from one subject to another Parviainen *et al* (1950), also using Heard and Sobel s method studied the excretion of reducing steroids in normal and abnormal pregnancy They found that in normally pregnant women the readings were on the average approximately twice as high as those in non pregnant subjects In cases of pre eclamptic toxæmia the urinary excretion figures were very variable and appeared to bear no relationship to the clinical features of the disease

### 3 Formaldehydogenic Steroids

Estimations of this group of steroids in clinical conditions have been reported by numerous workers including Daughaday *et al* (1948 *b*), Corcoran and Page (1948) Read *et al* (1950), King and Mason (1950) and Devis (1954)

(*a*) Normal Non pregnant Subjects —Daughaday *et al*

(1948 *b*) found that in normal adults of both sexes the amount of formaldehydogenic corticosteroids excreted lay between 1 and 1 6 mg per twenty four hours Read *et al* (1950) were able to demonstrate the presence of this material in the urine of newly born infants and showed that in the second week of life the excretion rose to a level not much lower than that obtained in adults The administration of ACTH to young infants resulted in an increased output of formaldehydogenic steroids King and Mason (1950) in a careful study, determined the excretion of this group of steroids in healthy children aged from one to fifteen years Values increased by 0 01 mg per twenty four hours for each increase in age of one year , in the older children the levels were very similar to those found in adults

(*b*) Disease States —The results obtained in various diseases show the same general trends as those found by Talbot *et al* (1951), using the copper reduction method In patients with Addison s disease Daughaday *et al* (1948 *b*) found abnormally low titres the levels usually being less than 0 65 mg per twenty four hours Readings below the normal range were also found in cases of panhypopituitarism, thyrotoxicosis and myxœdema On the other hand patients with Cushing s syndrome excreted abnormally large quantities, and in one case reported by Daughaday *et al* (1948 *b*) the output of formaldehydogenic steroids was 23 mg per twenty four hours

(*c*) Normal and Abnormal Pregnancy —Tobian (1949) estimated the excretion of formaldehydogenic steroids under these conditions The figures obtained in normally pregnant women were in the same range as in normal non pregnant individuals Elevated values were however, found in a proportion of patients with pre eclamptic toxæmia and were especially frequent in subjects showing œdema There was no apparent correlation between the excretion of formalde hydogenic steroids on the one hand and the degree of hypertension or albuminuria on the other

Venning *et al* (1949) have compared the results obtained by a biological method of assay of urinary corticosteroids with those found by two chemical assay methods The biological test depended on the deposition of glycogen in the liver of adrenalectomised mice while the two chemical procedures

were those involving copper reduction and formaldehyde formation In most of the normal individuals studied the copper reduction method yielded higher values than the formaldehydogenic method Many examples were found both in normal and abnormal conditions in which there was considerable disagreement from the quantitative point of view between the estimates obtained by bio assay on the one hand and by the two chemical assays on the other These divergent results, which can be explained on a methodological basis emphasise the necessity for the abandonment of these three assay methods for urinary corticosteroids and for their replacement in clinical investigations by more reliable procedures

## 4 Steroids determined by the Methods of Norymberski *et al*

### (a) 17 ketogenic Steroids

(i) Normal Non pregnant Subjects—The urinary excretion of 17 ketogenic steroids in normal individuals has recently been studied by a small number of workers in Europe and the United Kingdom including Norymberski *et al* (1953) Diczfalusy *et al* (1955), Prunty (1956) and Moxham and Nabarro (1956) In their original paper Norymberski *et al* (1953) reported that in normal men the figures ranged from 9 6 to 19 2 mg per twenty four hours with a mean value of 13 2 In normal women the output was generally lower the readings lying between 4 6 and 13 4 mg per twenty four hours with a mean figure of 8 9 In healthy children of both sexes varying in age from three to six years the values ranged from 2 3 to 3 8 mg per twenty four hours Figures quantitatively similar to those of Norymberski *et al* (1953) were obtained by subsequent workers However the total number of determinations so far reported in the literature is as yet too small to establish with certainty the upper and lower limits of urinary 17 ketogenic steroid excretion in healthy subjects at different ages

(ii) Disease States—At the time of writing little information is available in the literature on the excretion of 17 ketogenic steroids in pathological conditions Estimations in a small number of patients with endocrine diseases have been reported by Diczfalusy *et al* (1955) Prunty (1956) and Moxham and

Nabarro (1956) Very low readings have been obtained in cases of untreated Addison's disease, in panhypopituitarism and in patients subjected to hypophysectomy In the few cases of Cushing's syndrome so far studied the values have usually been considerably higher than those encountered in healthy subjects

Norymberski *et al* (1953) found that in patients with rheumatoid arthritis and with ankylosing spondylitis the output of urinary 17 ketogenic steroids was appreciably lower than in normal individuals In the male cases with ankylosing spondylitis the figures lay between 6 2 and 12 8 mg per twenty four hours In male patients with rheumatoid arthritis the range was from 7 3 to 8 4 mg per twenty four hours and in female patients with the same disease the levels varied from 3 1 to 10 8 mg per twenty four hours Treatment of rheumatic subjects with salicylates or with phenylbutazone did not affect the excretion of urinary 17 ketogenic steroids A great increase in the output of these steroids was noted in urine specimens obtained immediately after surgical operations

(iii) NORMAL AND ABNORMAL PREGNANCY —Scandrett (1957) showed that in normally pregnant women approaching term the excretion of 17 ketogenic steroids was approximately twice that in non pregnant subjects, following delivery a rapid fall in output occurred In a small number of patients with pre eclamptic toxæmia Scandrett (1957) found excretion values appreciably higher than those in normally pregnant women These observations suggest that the secretion of adrenocortical hormones increases as pregnancy advances and that it is especially high in pre eclamptic toxæmia

(iv) URINARY 17 KETOGENIC STEROID ASSAYS AS AN INDEX OF ADRENOCORTICAL FUNCTION —Prunty (1956) has investigated the functional activity of the adrenal cortex in man by measuring the urinary excretion of 17 ketogenic steroids and of 17 ketosteroids before and after the administration of a standard dose of ACTH The total duration of the test was six days On the first two days base line levels were determined, subsequently ACTH was injected intramuscularly in a dosage of 20 clinical units twice per day for four days

In patients with Addison's disease it was found that the excretion of 17 ketogenic steroids was abnormally low during

the control period and that no rise in output occurred after the administration of ACTH In cases of Cushing's syndrome basal levels of 17 ketogenic steroids were often above the normal range and a sharp rise in excretion occurred after the injection of the hormone Studies were also made in a group of patients with a condition which has been termed *basal hypo adrenal corticalism* In cases of this type who are usually suspected on clinical grounds to be suffering from either Addison's disease or panhypopituitarism adrenocortical function is subnormal and the nutritional status is often poor Such individuals show abnormally low levels of urinary 17 ketogenic steroids during the control period but give a rapid and large increase in urinary steroid output after the administration of ACTH Prunty (1956) has suggested that in patients with basal hypo adrenal corticalism the level of circulating ACTH is too low to maintain normal steroid excretion but is not low enough to cause adrenocortical atrophy sufficient to produce a failure of response to ACTH

### (*b*) Total 17 hydroxycorticosteroids

Moxham and Nabarro (1956) and Borth *et al* (1957 *a*) have recently studied the excretion of this group of steroids in normal individuals The results obtained by Borth *et al* (1957 *a*) in approximately 170 subjects are shown in Figs 56 and 57

It will be noted that the mean excretion in males at different ages tends to be higher than that in females In both sexes the excretion rises sharply at the time of puberty and reaches a maximum at about the age of thirty With advancing years the excretion of total 17 hydroxycorticosteroids falls progressively

Borth *et al* (1957 *b*) have also estimated the urinary excretion of total 17 hydroxycorticosteroids during the normal menstrual cycle Cyclic variations in output were not observed and the authors concluded that the adrenocortical hormones played little or no part in regulating the mechanisms which govern the rhythmic events occurring during the normal menstrual cycle

Appleby and Norymberski (1957) have found that the output of this group of steroids increases gradually during normal pregnancy and attains a maximum at the ninth month of gestation Following parturition the excretion decreases and

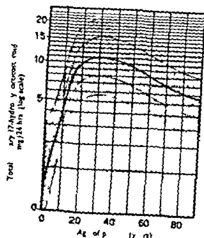



Fig 56

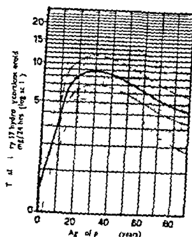



Fig 57

Fig 56 —Urinary excretion of total 17 hydroxycorticosteroids in healthy male subjects of average body weight and height The continuous line represents the mean excretion value the shaded area represents the fiducial range (P=0 95) (From Borth et al 1957 a)

Fig 57 —Urinary excretion of total 17 hydroxycorticosteroids in healthy female subjects of average body weight and height The continuous line represents the mean excretion value the shaded area represents the fiducial range (P=0 95) (From Borth et al 1957 a)

at the end of the first week of the puerperium the levels have returned to those normally encountered in non pregnant subjects

(c) **17 hydroxy-20-keto-21 deoxy *Steroids*** (21 deoxy ketols)

One of the most important members of this group of steroids from the quantitative point of view is 17α hydroxy pregnanolone Dobriner *et al* (1951) stated that this substance was excreted by patients suffering from rheumatoid arthritis but was not present in the urine of normal individuals This finding was not confirmed in subsequent investigations Recently Appleby and Norymberski (1955) have studied the urinary excretion of 21 deoxyketols in normal subjects and in patients with rheumatoid arthritis They found that the mean excretion of this group of steroids did not differ significantly in the two groups of patients and concluded that an abnormally high excretion of 21 deoxyketols was not characteristic of cases of rheumatoid arthritis

Appleby and Norymberski (1957) have shown that in

normal pregnancy the excretion of 21 deoxyketols rises progressively At the fourth month the mean figure was 0 58 mg per twenty four hours and by the ninth month the output had increased to 1 34 mg per twenty four hours After parturition a rapid decrease in excretion occurred and within approximately one week of delivery the levels were similar to those normally found in non pregnant subjects Detailed studies of the excretion of 21 deoxyketols in the various types of abnormal pregnancy have not yet been conducted The results of such an investigation will be awaited with interest

5 **17 21 dihydroxy-20 keto corticosteroids** (Porter Silber chromogens)

(*a*) **Estimations in Blood**

Most investigators have employed the technique described by Nelson and Samuels (1952) or some modification thereof (see p 264)

(i) Normal Non pregnant Subjects – Nelson *et al* (1951) found that in normal individuals of both sexes the concentration of 17 21 dihydroxy 20 keto corticosteroids ranged from 4 to 10 μg per 100 ml of whole blood The administration of ACTH to normal subjects caused a sharp increase in the blood concentration of this group of steroids With a single intra venous injection of ACTH the steroid level attained its maximum in approximately one hour and returned to the pre injection value within three hours When the hormone was administered intramuscularly the maximal level was reached in three hours and the concentration had returned to normal in four to six hours In the opinion of Nelson *et al* (1951) the continuous intravenous infusion of ACTH was the most efficient mode of exhibition of the hormone This resulted in a steady rise in blood corticosteroid concentration to levels which at the end of twenty four hours were higher than those achieved by any other method of administration

Gemzell (1953 1954) has conducted extensive studies with the Nelson Samuels technique in a variety of clinical conditions In a series of healthy young adults of both sexes the mean plasma concentration of 17 21 dihydroxy 20 keto corticosteroids was 6 6±0 9 μg[1] per 100 ml Under normal conditions the level of these steroids showed some diurnal variation the titre

[1] Standard deviation of a single observation

being relatively higher during the first part of the day and decreasing to a minimum by the late evening

(ii) DISEASE STATES—In patients with Addison's disease who were not receiving therapy, Nelson *et al* (1951) showed that the concentration of 17 21 dihydroxy 20 keto cortico steroids fell to zero In cases of rheumatoid arthritis or of acute rheumatic fever the concentrations were within normal limits, while in patients suffering from severe or fulminant diseases an increased blood concentration of these hormones was demonstrable only in the terminal phases of the illness In cases of Cushing s syndrome irrespective of ætiology, the titre is generally above the normal range

Franksson *et al* (1954) have studied the plasma levels of 17 21 dihydroxy 20 keto corticosteroids in patients after major *surgery All cases showed elevated titres in the immediate* post operative period, in uncomplicated cases the levels fell to normal within approximately thirty six hours In patients showing post operative shock very high readings were found and these persisted as long as the shock was present In *individuals with prostatic carcinoma who had been treated* by bilateral adrenalectomy Gemzell and Franksson (1953) showed that the daily administration of 50 mg of cortisone acetate by the oral route was sufficient to maintain the levels of plasma corticosteroids within the normal range However, if the cortisone therapy was withdrawn the blood hormone content fell precipitously and within forty to sixty hours zero levels were encountered

(iii) NORMAL PREGNANCY—Various workers including Gemzell (1953), Bayliss *et al* (1955) and Robinson *et al* (1955) have determined the plasma concentration of 17 21 dihydroxy 20 keto-corticosteroids throughout normal pregnancy The results obtained by Gemzell (1953) are shown in Fig 58

It will be noted that the plasma corticosteroid concentration increased significantly with the duration of pregnancy and that in the third trimester the values were approximately four times those found in normal non pregnant subjects Immediately following parturition a further rise occurred and at this time levels greater than 30 μg per 100 ml were frequently encountered The mean post partum concentration of corticosteroids in primiparæ was significantly higher than that in multiparæ but no correlation could be demonstrated between

the corticosteroid levels on the one hand and the age of the mother, duration of labour and weight and sex of the infant on the other Soon after parturition a fall in plasma corticosteroid levels occurred and approximately one week after delivery the titres were again within the range found normally in non pregnant subjects

Bayliss *et al* (1955) in a careful investigation undertook

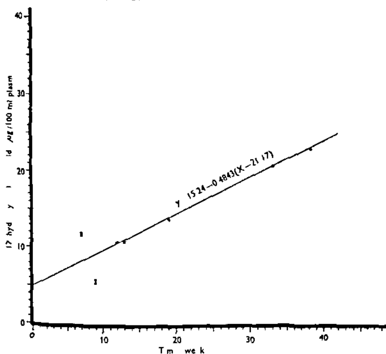



FIG 58

Correlation between the concentration of 17 21-dihydroxy 20-keto-corticosteroids in the blood of pregnant women and the duration of pregnancy in weeks Concept on is assumed to have occurred at zero time (*From Gemzell* 1953)

serial estimations of plasma 17 21 dihydroxy 20 keto corticosteroids in thirty normally pregnant women Their results which from the quantitative point of view agree reasonably well with those of Gemzell (1953) are shown in Fig 59

It will again be seen that the mean plasma corticosteroid level rose progressively during pregnancy and that after delivery the titre fell returning to normal after approximately one week Bayliss *et al* (1955) noted that the readings varied greatly

from one patient to another at comparable times of pregnancy but were unable to demonstrate any clinical differences between patients who showed high levels and those in whom the titre

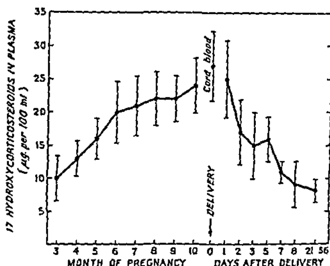



FIG 59

Mean plasma concentrations of 17 21-dihydroxy 20-keto-corticosteroids throughout normal pregnancy and during the puerperium Vertical bars indicate the standard deviation of a single observation (*From Bayliss et al* 1955)

was relatively low In the opinion of these workers the most probable source of the increased corticosteroid production during pregnancy is the maternal adrenal gland, it was felt that if the placenta was the major source of these hormones the post partum fall in plasma concentration would be much more rapid than that actually observed

### (*b*) Estimations in Urine

In such estimations most workers have employed the technique described by Reddy *et al* (1952) or some modification thereof (see p 259)

(*i*) NORMAL SUBJECTS —*The normal range of excretion* has varied widely in the hands of different investigators Liddle *et al* (1954) reported values ranging from 5 to 16 mg per twenty four hours with a mean figure of 10 mg, while Jenkins *et al* (1955) found levels varying between 1 and 10 mg per twenty four hours with a mean excretion figure of 5 2 mg These divergent results can probably be explained on a

methodological basis Recently Moxham and Nabarro (1956) have reported that a good correlation exists between the excretion of 17 21 dihydroxy 20 keto corticosteroids on the one hand and the output of urinary 17 ketogenic steroids on the other

(ii) DISEASE STATES —In untreated Addison s disease and in patients after bilateral adrenalectomy Liddle *et al* (1954) found values ranging from zero to 5 mg per twenty four hours *In cases of Cushing s syndrome on the other hand the readings* lay between 15 and 70 mg per twenty four hours

Ely *et al* (1955) have studied the output of 17 21 dihydroxy 20 keto corticosteroids in children with acute rheumatic fever before during and after various types of treatment In untreated patients the levels of excretion were similar to those found in normal children Treatment by cortisone caused a rise in corticosteroid excretion and the rise was proportional to the dosage of the hormone administered On cessation of hormonal therapy a rapid fall in corticosteroid output occurred

(iii) *URINARY DETERMINATIONS OF 17 21 DIHYDROXY 20* KETO CORTICOSTEROIDS AS A TEST OF ADRENAL FUNCTION — Liddle *et al* (1954) have studied adrenocortical activity in normal and pathological conditions by estimating the excretion of these steroids after the intravenous infusion of a standard dose of ACTH over an eight hour period These workers claimed that this test would differentiate patients with cortical hyper or hypo function from normal individuals and that in this respect it was more reliable than methods depending on 17 ketosteroid excretion electrolyte excretion and eosinophil counts In subjects with normal adrenal function the urinary output of corticosteroids increased approximately threefold over control levels in the twenty four hours following the ACTH administration In cases of adrenal hyperfunction from what ever cause, the maximal excretion was on all occasions greater than that of normal individuals while in patients with Addison s disease little or no rise in urinary corticosteroid excretion occurred

## 6 Aldosterone

In 1950 Deming and Luetscher reported that chloroform extracts prepared from the urine of patients with oedema

caused sodium retention in adrenalectomised rats while similar extracts from normal subjects failed to elicit this effect Luetscher and his collaborators (Luetscher *et al*, 1952, 1954) conducted a thorough investigation into the nature of this salt retaining hormone and demonstrated that it was identical with aldosterone, the hormone recently isolated by Simpson *et al* (1953) from ox adrenal extracts

During the past few years various workers have attempted to measure the urinary excretion of aldosterone in normal and pathological conditions in man Although the assay methods now available are far from satisfactory from the quantitative point of view, clinical information of some interest has already been obtained The field of aldosterone assay is at present a very fluctuant one in which rapid advances can be expected as more reliable methods of estimation are developed For a critical appraisal of the status of aldosterone assays in clinical practice up to 1955 the reader is referred to a review article by Gaunt *et al* (1955)

As emphasised by Bartter *et al* (1956) Neher and Wettstein (1956) and others, the aldosterone content of urine in health and disease is markedly affected by such factors as the state of hydration of the patient and the dietary intake of sodium and potassium These observations make it essential to obtain accurate information on a patient's water and electrolyte balance before conducting urinary assays of aldosterone

(*a*) NORMAL NON PREGNANT SUBJECTS —Variable results have been reported on the excretion of urinary aldosterone in normal individuals This is scarcely surprising in view of the unsatisfactory nature of the assay methods at present in use One of the more detailed studies is that of Venning *et al* (1956) These workers used a bio assay method depending on the excretion of sodium in adrenalectomised rats, the urine was hydrolysed by β-glucuronidase and extracted with chloroform Using this technique it was possible to demonstrate aldosterone activity in both normal males and females Serial determinations showed that there was considerable day to day variation in the excretion of aldosterone in the same individual

Probably the most reliable estimates yet published of the urinary aldosterone content in healthy male subjects are those of Ayres *et al* (1956) who used a chemical assay method which is described on page 261 These workers reported that the

mean urinary excretion of aldosterone in nineteen subjects was 10 5 μg per twenty four hours and that the figures ranged from 4 6 to 18 9 μg per twenty four hours

(*b*) DISEASE STATES —(i) NEPHROTIC SYNDROME —All investigators report abnormally high excretion values in patients with this disease According to Luetscher *et al* (1954) the quantities of aldosterone present in the urine of such patients are sufficiently large to make this type of human urine the best natural source of the hormone Present evidence indicates that the rate of excretion of aldosterone is not related to the urine volume and is independent of the degree of proteinuria (Luetscher and Johnson 1954) Following clinical improvement with diminution in the degree of œdema the urinary output of aldosterone decreases

(ii) CARDIAC FAILURE WITH ŒDEMA —Abnormally high titres of aldosterone have been reported in the majority of cases studied (Singer and Wener 1953 Cope and Garcia Llaurado 1954) The ætiology of the cardiac failure appears to be of little importance in relation to the quantities of aldosterone excreted

(iii) ESSENTIAL HYPERTENSION —Genest *et al* (1956) in a careful investigation reported that patients with essential hypertension excreted significantly larger quantities of urinary aldosterone than did healthy subjects These workers put forward the view that one of the ætiological factors in this disease might be a mild but long continued hypersecretion of aldosterone This interesting suggestion requires further study

(iv) CIRRHOSIS OF THE LIVER —In portal cirrhosis complicated by ascites very high titres are generally found (Chart and Shipley 1953, Pechet *et al* 1954) According to Chart and Shipley (1953) the readings may lie within the normal range when ascites is absent

(v) ADDISON S DISEASE —In a case reported by Luetscher and Axelrad (1954) no activity was detected in urine extracts Zero levels were also encountered in two patients after bilateral adrenalectomy These findings were taken as evidence that the adrenal cortex was the site of production of the salt retaining hormone

(vi) PANHYPOPITUITARISM —In two cases investigated by Luetscher and Axelrad (1954) the readings were within the

normal range, although the excretion of pituitary gonadotrophins was abnormally low This observation supports the currently held view that the sodium regulating function of the adrenal cortex is largely independent of the pituitary

(vii) 'PRIMARY ALDOSTERONISM'—This term was first used by Conn (1955) to denote a syndrome characterised by high levels of aldosterone in urine, hypokalæmia, hypernatræmia alkalosis, polyuria, intermittent tetany, muscular weakness and hypertension Patients with a similar clinical picture have been subsequently described by Mader and Iseri (1955), Chalmers *et al* (1956) and van Buchem *et al* (1956) In the few cases so far reported the adrenal glands have shown evidence either of tumour or of hyperplasia, and it has been suggested that the condition results from an increased production of aldosterone by the adrenal cortex Further work is necessary to establish whether this syndrome definitely exists as a separate entity

(*c*) NORMAL AND ABNORMAL PREGNANCY —The urinary excretion of aldosterone during normal pregnancy has been investigated by Gordon *et al* (1954), Martin and Mills (1956) and Venning and Dyrenfurth (1956) In a recent paper Venning and Dyrenfurth (1956) have shown that the hormone is excreted in increasing amounts during the gestation period and that in the second and third trimesters the readings are much higher than those in non pregnant individuals After delivery a rapid fall to non pregnant levels occurs According to Venning and Dyrenfurth (1956) aldosterone occurs in urine throughout pregnancy mainly in the conjugated form as aldosterone glucuronide

Reports are at present conflicting with respect to the excretion of aldosterone in patients with pre eclamptic toxæmia Further information on this important subject must await the development of more reliable assay methods for the hormone

## SUMMARY AND CONCLUSIONS

Corticosteroids in urine should be estimated by chemical rather than by biological methods At the time of writing there is no assay method which is completely satisfactory for the quantitative determination of these hormones and their metabolites in urine

Of the chemical techniques at present available, probably the best for clinical purposes are those developed by Norymberski *et al* Methods depending on the reducing or formaldehydogenic properties of adrenocortical steroids should no longer be used It is probable that in the future assay methods for corticosteroids in body fluids will depend either on paper chromatographic techniques or on procedures employing radio-active steroids

Methods for the quantitative determination of aldosterone in urine are at present at a very early stage of development Much further work is necessary before such techniques can be used routinely in the clinical field

The estimation of corticosteroids in blood presents a difficult problem from the methodological point of view The technique recently described by Weichselbaum *et al* (1953) for the separate determination of the free and conjugated adrenocortical steroids in plasma merits further application in clinical studies

Corticosteroid assays in body fluids may be of diagnostic value in conditions of stress, and in such diseases as Cushing s syndrome, the adrenogenital syndrome Addison s disease and panhypopituitarism The estimation of corticosteroids in blood and urine after the administration of ACTH forms a useful test of adrenocortical function in man

In normal pregnancy the urinary excretion and plasma concentration of corticosteroids rise progressively Soon after delivery a rapid fall in the titre occurs and within a few days the levels are in the range normally encountered in non pregnant subjects

The aldosterone content of urine is influenced markedly by the state of hydration and the electrolyte balance of the patient Abnormally high levels of excretion of aldosterone have been reported in such conditions as the nephrotic syndrome cardiac failure with œdema and portal cirrhosis with ascites

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# CHAPTER XII

## 17-Ketosteroids and Androgens

IN this chapter three main topics will be discussed These are

I The urinary neutral 17 ketosteroids
II Neutral 17 ketosteroids in blood
III Androgens in blood and urine

### I THE URINARY NEUTRAL 17 KETOSTEROIDS

### INTRODUCTION

In 1931 Butenandt isolated from male urine a crystalline steroid which he termed androsterone This substance was the first of the group of compounds now known as the neutral urinary 17 ketosteroids, and since Butenandt s discovery many structurally similar compounds have been isolated from human urine under normal and pathological conditions For a detailed account of the chemical properties of the various 17 ketosteroids the reader is referred to publications by Fieser and Fieser (1949) Engel (1954) and Dorfman and Shipley (1956)

All 17 ketosteroids have in common an oxygen atom attached at C 17, all are derivatives of two parent hydro carbons, 5α androstane and 5β androstane (ætiocholane) the formulæ of which are shown in Fig 60

It will be noted that these two substances differ only in the spatial configuration of the hydrogen atom at C 5

In Fig 61 are shown the formulæ for eight of the more important urinary 17 ketosteroids

The first four compounds are unsubstituted at C 11 while the last four have an oxygen substituent in this position in the form of either an oxo (=O) or a hydroxyl (−OH) group Isomerism at C 3 is encountered relatively frequently in this group of steroids This is well illustrated by comparing the structure of the two compounds androsterone and *epi*andro sterone In androsterone the hydroxyl group at C 3 is on the

opposite side of the molecule to the methyl group at C 10 This is termed the *trans* or α configuration and is designated by a broken line In *epi*androsterone on the other hand the hydroxyl group at C 3 is on the same side of the molecule as is the methyl group at C 10 This is the *cis* or β configuration and is represented by a solid line *Epi*androsterone and dehydro*epi*androsterone are the principal 17 ketosteroids with the 3β OH group and the latter compound forms the main part of the so called 3β hydroxy fraction However, the major portion of the total 17 ketosteroids excreted in urine show the α configuration of the OH group at C 3 and from the quantitative point of view the two most important

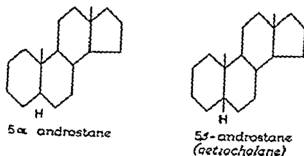



Fig 60
Structural formulæ of 5α androstane and 5β androstane (ætiocholane)

compounds in the 3α hydroxy fraction are androsterone and 5β androstanolone

It should be noted that the œstrogenic steroid œstrone is also a 17 ketosteroid However this compound, due to the phenolic structure of ring A is acidic in nature and is removed from urinary extracts when these are washed with alkali Accordingly œstrone is not included in the term neutral 17 ketosteroids and will not be considered in the present chapter Its structural formula is shown on page 155

The various urinary 17 ketosteroids differ to a considerable extent in their biological activities This subject has recently been reviewed by Dorfman and Shipley (1956) The most widely used assay method has been that depending on the capon s comb (see p 313) Using this test androsterone has been shown to possess approximately one sixth of the biological activity of the male sex hormone testosterone while *epi* androsterone and dehydro*epi*androsterone are respectively about

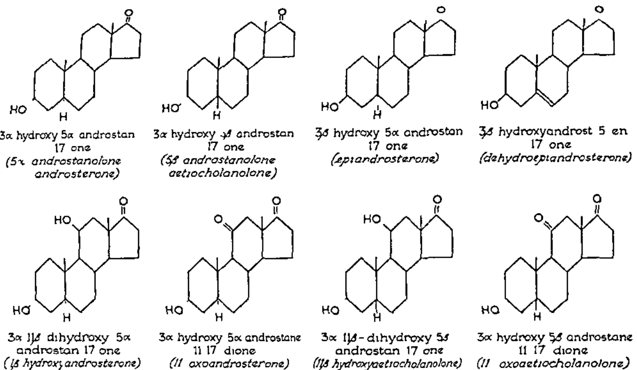



Fig. 61

Structural formulæ of eight of the more important urinary 17 ketosteroid

one tenth and one third as active as androsterone The compound 5$\beta$ androstanolone, on the other hand, has not been shown to possess biological activity From these and other observations it is apparent that the term 17 ketosteroid is by no means synonymous with the term androgen

The 17 ketosteroids are excreted in urine in the *conjugated* rather than the *free* form Probably the majority of compounds are conjugated with glucuronic acid and sulphuric acid and are excreted as glucuronides or sulphates It is, however, conceivable that other as yet unidentified conjugates are also present in human urine

## SOURCES IN THE BODY

It is generally believed that the urinary 17 ketosteroids arise from precursors which have been secreted by the adrenal cortex, by the testes, and possibly to a small extent by the ovaries In men approximately one third of the total urinary 17 ketosteroids are thought to represent metabolites of the testicular hormone testosterone while the remaining two thirds are derived from steroids which have been elaborated by the adrenal cortex In women who usually excrete smaller quantities than men, the main source of production is the adrenal cortex

It should be emphasised that the urinary 17 ketosteroids which are of adrenal origin arise in two different ways being formed partly by oxidative removal in the tissues of the side chain of certain of the C 21 steroids and partly from C 19 steroids which have been secreted by the adrenal cortex itself

## THE CHEMICAL ESTIMATION OF THE URINARY NEUTRAL 17 KETOSTEROIDS

The main steps in the majority of methods in clinical use are as follows (1) Hydrolysis of the 17 ketosteroid conjugates by acid in order to break down the conjugates and thereby to liberate the free steroids for extraction, (2) extraction of the free compounds with organic solvents *e g*, carbon tetrachloride, ethylene dichloride ether or benzene, (3) removal of the acidic material from the extracts by washing with alkali leaving behind the neutral fraction as residue (4) development

of the colour reaction after dissolving the dry residue of the neutral fraction in alcohol, (5) correction for interfering chromogenic material in the final extract by the use of an appropriate formula such as that described by Talbot *et al* (1942)

It should be borne in mind that at present conditions for the hydrolysis of urinary 17 ketosteroid conjugates are not entirely optimal Most workers carry out the hydrolysis by boiling the urine for ten to thirty minutes after the addition of hydrochloric acid or sulphuric acid It is unlikely that with this procedure the yield of all the 17 ketosteroids is strictly quantitative, *e g*, Gallagher and Moulton (1944) and Lieberman and Teich (1953) have reported that with acid hydrolysis some loss of steroids of the 3$\beta$ hydroxy fraction particularly dehydro*epi*androsterone may occur Recently attempts have been made to use the enzyme $\beta$ glucuronidase for the hydrolysis of urinary 17 ketosteroid conjugates In view of the relatively slow action of preparations of this enzyme, it is probable that this form of hydrolysis will not be suitable for routine assays in the clinical field

Another factor which must be considered during the quantitative determination of 17 ketosteroids in urine is the formation of artifacts These substances arise as a result of reactions which occur during the processes of hydrolysis, extraction and isolation, they may cause either an under estimate or an over estimate of the true 17 ketosteroid concentration in urine The subject of artifact formation has been studied intensively by Dorfman and his co workers (Dorfman and Ungar, 1953, Ungar and Dorfman 1953 Dorfman and Shipley 1956) For further information the reader is referred to the original articles

A number of *colorimetric methods* are available for the final determination of the urinary 17 ketosteroids Most of these are based on the procedure originally described by Zimmermann (1935) This reaction depends on the fact that steroids containing the group —CO—CH —will give a purple colour when treated with *m* dinitrobenzene in the presence of alkali Under the conditions usually employed the colour measured is due mainly to the 17 ketosteroids although steroids with ketonic groups at C-3 and C 20 will also take part in the reaction and will produce colours of a lower intensity In the urine of

one tenth and one third as active as androsterone The compound 5β androstanolone, on the other hand, has not been shown to possess biological activity From these and other observations it is apparent that the term 17 ketosteroid is by no means synonymous with the term androgen

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techniques are reasonably precise in addition to being simple and rapid, and appear to be ideally suited for routine application in the clinical field

In the procedures described above the urinary 17 keto steroids are determined in the unconjugated form Very recently Kellie and Wade (1956) have developed a technique in which the steroids can be estimated in conjugated form as glucuronides and sulphates The application of this technique to clinical problems will be awaited with interest

## FRACTIONATION OF URINARY 17 KETOSTEROIDS

### 1 Separation by Girard's Reagent

17 Ketosteroids can be fractionated by treatment of the total neutral fraction of urine with the Girard reagent T (trimethylammoniumacetohydrazide chloride) This reagent will separate the ketonic neutral fraction containing the 17 ketosteroids from non ketonic neutral compounds The fractionation depends on the fact that Girard s reagent T forms water soluble derivatives with the 17 ketosteroids so that the ketones can be separated from the non ketonic material by distribution between water and ether at low temperatures Girard separation has proved of little practical value in clinical studies and is now seldom employed in laboratories conducting urinary 17 ketosteroid assays

### 2 Separation by Digitonin

Separation of the $3\alpha$ hydroxy and $3\beta$ hydroxy fractions in urine has been attempted by the use of digitonin (Talbot *et al* 1940, Dobriner *et al* 1948) This substance is capable of forming insoluble complexes with steroids showing a hydroxyl group with the $\beta$ configuration at C 3 but does not react with $3\alpha$ hydroxysteroids or with steroids with other substituents at C 3 Digitonin separation has been used in the clinical field particularly in the investigation of patients with adrenal hyperfunction some of whom excrete large quantities of $3\beta$ hydroxy 17 ketosteroids particularly dehydro*epi*androsterone

Separation by digitonin is not a very satisfactory technique from the quantitative point of view and therefore caution is necessary in the interpretation of results obtained by its use

non pregnant individuals the quantities of C 3 and C 20 ketones present are very small and it is unlikely that any interference with the colour reaction will occur However, during pregnancy relatively large amounts of C 20 ketones especially 5α pregnan 3α ol 20 one are excreted (Venning 1946) and it is probable that under these conditions the Zimmermann reaction may yield an over estimate of the true concentration of 17 ketosteroids present (see p 298)

Many modifications of the Zimmermann reaction have been proposed but the two most popular techniques in clinical investigations have been the methods developed by Callow *et al* (1938) and by Holtorff and Koch (1940) One or other of these two procedures in their original or slightly modified form are now employed in most centres In 1951 the Clinical Endocrinology Committee of the Medical Research Council concluded that the Callow Zimmermann method was the best of the techniques available and recommended that this procedure should be adopted in all laboratories undertaking assays of urinary 17 ketosteroids for clinical purposes For details of the actual method suggested the original article should be consulted

Pincus (1943) has stated that the urinary 17 ketosteroids can be determined by a colour reaction with antimony trichloride However this technique does not measure dehydro*epi*androsterone an important compound in relation to clinical diagnosis and for this reason is probably inferior to procedures based on the Zimmermann reaction

Wolfe *et al* (1940) and Morris and his collaborators (Barnett *et al* 1946 *a b* Butt 1950) have used *polarographic* rather than colorimetric methods for urinary 17 ketosteroid determinations When the same urinary extracts are analysed both polarographically and by the Callow Zimmermann method a good correlation is generally obtained (Butt *et al*, 1951) Polarographic techniques are probably reliable and reproducible but have not yet had such a wide application in clinical studies as methods depending on colorimetry

A number of *micro methods* have been proposed for 17 ketosteroid assays (Drekter *et al*, 1947 1952 Hamburger and Rasch 1948, Vestergaard 1951) In these procedures a few millilitres of urine are hydrolysed and extracted and the final extract is subjected to colorimetric analysis Such

techniques are reasonably precise in addition to being simple and rapid and appear to be ideally suited for routine application in the clinical field

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Separation by digitonin is not a very satisfactory technique from the quantitative point of view and therefore caution is necessary in the interpretation of results obtained by its use

For example, it has been reported (Engel, 1954) that, when the excretion of 3β hydroxysteroids is high as in patients with adrenocortical tumours, incomplete precipitation may occur In view of the development of more efficient chromatographic techniques for the estimation of the individual 17 ketosteroids in urine it is unlikely that digitonin precipitation will continue to be used in clinical studies

## 3 Separation by Chromatography

Much fundamental work on the fractionation of the individual urinary 17 ketosteroids by chromatographic methods was performed by Dingemanse *et al* (1946) and by Dobriner *et al* (1948) In the technique described by Dingemanse *et al* (1946) adsorption chromatography on alumina columns was employed and the 17 ketosteroids were separated into eight main fractions as judged by peaks on the chromatogram

Methods similar in principle to that of Dingemanse *et al* (1946), but employing a more convenient micro scale were later developed by Zygmuntowicz *et al* (1951), Pond (1951) *Johnsen (1956 a) and others* One of the more elegant modifications of the original technique is that recently described by Plantin and Birke (1954) These workers also employed alumina columns but subsequently identified the individual urinary ketosteroids by means of infra red spectroscopy

Kochakian and Stidworthy (1952), Savard (1953) and Rubin *et al* (1954) have reported effective separation of 17 ketosteroids by paper chromatographic means The zones containing the 17 ketosteroids were detected by spraying the paper with alkaline *m* dinitrobenzene in order to produce the characteristic Zimmermann colour

Using such fractionation techniques attempts have been made in recent years to study the pattern of urinary 17 ketosteroid excretion in patients with various forms of endocrine disorders and to compare the values obtained with those in normal individuals

## THE URINARY EXCRETION OF NEUTRAL 17-KETOSTEROIDS IN NORMAL SUBJECTS

The excretion of total neutral 17 ketosteroids in the urine of normal individuals has been estimated by numerous workers

in the last two decades Among the more comprehensive studies are those of Callow *et al* (1940), Fraser *et al* (1941), Forbes *et al* (1947), Hamburger (1948) and Plantin and Birke (1954) In most investigations the final colorimetric determination has depended on the Zimmermann reaction although in a few publications such as those of Barnett *et al* (1946 *a b*) a polarographic method has been used In general the modification of the Zimmermann reaction described by Callow *et al* (1938) has been more popular than that of Holtorff and Koch (1940)

A few studies have been reported in which estimations of the 3β-hydroxy ketosteroids have been undertaken using digitonin separation (Talbot *et al* 1940 Dobriner *et al* 1948 Dingemanse *et al* 1952) Usually in normal subjects this fraction has been found to be less than 20 per cent of the total 17 ketosteroids present Various workers including Pond (1954) Rubin *et al* (1954) Plantin and Birke (1954) and Johnsen (1956 *a*) have fractionated the urinary 17 ketosteroids by chromatographic means and have thus been able to study the excretion of the individual compounds in health and disease These results will be considered on page 308 The present section of the chapter (pp 295 308) deals with the excretion of total rather than of individual urinary 17 ketosteroids

**1 Normal Men**

In men between the ages of twenty and forty the majority of values lie between 12 and 17 mg per twenty four hours According to Mason and Engstrom (1950) the lower limit of normal is approximately 6 mg and the upper limit 25 mg Fig 62 which is taken from a paper by Hamburger (1948) shows the excretion of 17 ketosteroids in 137 healthy male subjects varying in age from 3 to 102

It will be noted that the mean output of 17 ketosteroids rises rapidly at the time of puberty and reaches a maximum at about the age of twenty five With advancing years the values fall progressively and by the seventh decade the mean excretion is only approximately half of that in the third and fourth decades It is reasonable to assume that the output of urinary 17 ketosteroids in older male subjects results mainly from a reduction in the secretion of steroid hormone precursors by the adrenal cortex and the testes

Recently Hamburger (1954) has reported a study of the

For example, it has been reported (Engel, 1954) that, when the excretion of 3β hydroxysteroids is high as in patients with adrenocortical tumours, incomplete precipitation may occur. In view of the development of more efficient chromatographic techniques for the estimation of the individual 17 ketosteroids in urine it is unlikely that digitonin precipitation will continue to be used in clinical studies.

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## THE URINARY EXCRETION OF NEUTRAL 17-KETOSTEROIDS IN NORMAL SUBJECTS

The excretion of total neutral 17 ketosteroids in the urine of normal individuals has been estimated by numerous workers

## 2 Normal Non-pregnant Women

It is generally agreed that normal women tend to excrete smaller quantities of urinary 17 ketosteroids than do normal men of a comparable age group There is however a considerable overlap in the values for the two sexes In women

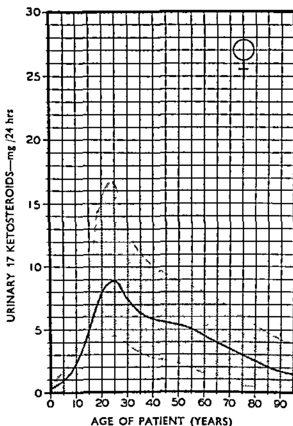



Fig 63

Urinary excretion of 17 ketosteroids in normal women The continuous line represents the mean excretion value The shaded area represents the range within which 97 to 98 per cent of the values fall (*From Hamburger* 1948)

between the ages of twenty and forty the majority of readings lie between 7 and 12 mg per twenty four hours In this age group the lower limit of normal is probably about 3 mg and the upper limit 20 mg Fig 63 which is also taken from a publication by Hamburger (1948) shows the excretion of

daily excretion of urinary 17-ketosteroids in a healthy male subject over a six year period from the age of forty four to fifty. It was found that the average daily excretion decreased by 1 mg per twenty four hours in the course of the six years. The total mean value during the period of study was

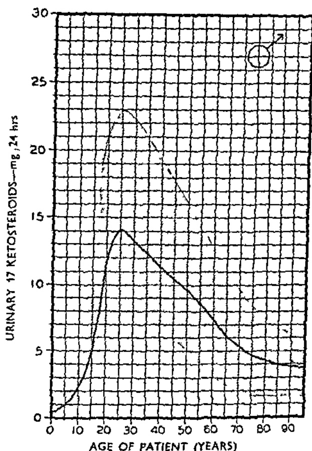



FIG 62

Urinary excretion of 17 ketosteroids in normal men. The continuous line represents the mean excretion value. The shaded area represents the range within which 97 to 98 per cent of the values fall. (*From Hamburger* 1948)

approximately 12 mg per twenty four hours and the levels ranged from 9 to 16.4 mg. Marked variations in excretion occurred from one day to another but no relationship was demonstrated between the quantities excreted on the one hand and the urine volume on the other

technique On the other hand the antimony trichloride reaction of Pincus is more specific for the 17 ketosteroids and presumably would not be affected to the same extent by the presence of 20 ketosteroids in urine

### 4 Children

In both male and female children below the age of six the urinary excretion of 17 ketosteroids is very low and is frequently less than 1 mg per twenty four hours (Figs 62 and 63) From the age of seven until puberty a gradual increase in output occurs and at the time of puberty the average figure in both sexes is about 9 mg per twenty four hours Normal adult levels are generally reached in the period between the ages of seventeen and twenty Since the urinary 17 ketosteroids arise from precursors secreted by the adrenal cortex and the testis the similarity in the levels excreted by boys and girls prior to puberty suggests that in this age period the adrenal cortex is the main site of production of these precursors

## THE URINARY EXCRETION OF NEUTRAL 17 KETOSTEROIDS IN PATHOLOGICAL CONDITIONS

### 1 Diseases of the Adrenal Cortex

Estimations of the total urinary 17 ketosteroids of the 3$\beta$ hydroxy fraction or of the individual compounds have been extensively used in the clinical field in an attempt to differentiate cases of adrenocortical tumour from those of adrenocortical hyperplasia Although in many instances such assays have proved of diagnostic value on other occasions the results obtained have been inconclusive

(*a*) ADRENOCORTICAL TUMOUR —Patients with these neoplasms may present with the clinical features either of *Cushing s syndrome* or of the *adrenogenital syndrome*[1] 17 Ketosteroid assays

[1] The term *Cushing s syndrome* will be used to denote a particular symptom complex the main features of which are a distinctive bodily habitus characterised by facial and abdominal obesity with relatively thin arms and legs purple striæ and ecchymoses hirsutism hypertension osteoporosis amenorrhœa diminished carbohydrate tolerance muscular weakness and occasionally polycythæmia and hypochloræmic alkalosis The syndrome occurs characteristically in young women but is also found in men and in children of both sexes Apart from the presence of hirsutism there is little evidence of masculinisation

The term *adrenogenital syndrome* will be used to describe a symptom-complex which is most frequently found in young women but which may occur in either sex at any time of life The main features are those of masculinisation *e g* excess muscularity enlargement of the larynx clitoral hypertrophy and hirsutism of the male type

urinary 17 ketosteroids in 127 healthy female subjects between the ages of two and ninety two

It will be observed that, as in the case of normal males, the mean output rises sharply with the onset of puberty and adolescence and reaches a maximum at about the age of twenty five Thereafter, according to Hamburger (1948), the curve for the 17 ketosteroid excretion in females is rather different from that in males Whereas in the latter a progressive fall occurs with advancing years, the female subjects show a relatively sharp decrease in output between the ages of twenty five and thirty five, fairly constant levels from thirty five to fifty five and a gradual fall in output after the age of sixty The urinary 17 ketosteroids in women arise almost exclusively from precursors secreted by the adrenal cortex, and it is reasonable to assume that the fluctuations at different ages represent, at least to some extent, variations in the secretory activity of this gland

A number of investigators including Werner (1941) Davis and Plotz (1956) and Borth *et al* (1957) have studied the 17 ketosteroid excretion throughout the normal menstrual cycle No definite cyclic fluctuations have been observed and the small fluctuations which do occur appear to bear no constant relationship to the various phases of the cycle

### 3 Normally Pregnant Women.

Venning (1946) has estimated the 17 ketosteroid excretion throughout normal pregnancy Two colorimetric assay methods were used, one depending on the Holtorff Koch modification of the Zimmermann reaction and the other on the antimony trichloride reaction of Pincus The material giving a reaction in the modified Zimmermann procedure increased gradually as pregnancy advanced while the material active in the antimony trichloride reaction remained relatively constant throughout the gestation period These divergent results can probably be explained on a methodological basis The modified Zimmermann procedure is not entirely specific for urinary 17 ketosteroids (see p 292) and is affected to some extent by the presence of 20 ketosteroids in urine extracts It is therefore probable that the increased excretion of $5\beta$ pregnan $3\alpha$ ol 20 one and of other urinary 20 ketosteroids as pregnancy advances is sufficient to account for the apparent rise in 17 ketosteroid output as measured by the Holtorff Koch

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(a) Adrenocortical Tumour —Patients with these neoplasms may present with the clinical features either of *Cushing s syndrome* or of the *adrenogenital syndrome*[1] 17 Ketosteroid assays

[1] The term *Cushing s syndrome* will be used to denote a particular symptom complex the main features of which are a distinctive bodily habitus characterised by facial and abdominal obesity with relatively thin arms and legs purple striæ and ecchymoses hirsutism hypertension osteoporosis amenorrhœa diminished carbohydrate tolerance muscular weakness and occasionally polycythæmia and hypochloræmic alkalosis The syndrome occurs characteristically in young women but is also found in men and in children of both sexes Apart from the presence of hirsutism there is little evidence of masculinisation

The term *adrenogenital syndrome* will be used to describe a symptom-complex which is most frequently found in young women but which may occur in either sex at any time of life The main features are those of masculinisation *e g* excessive muscularity enlargement of the larynx clitoral hypertrophy and hirsutism of the male type

urinary 17 ketosteroids in 127 healthy female subjects between the ages of two and ninety two

It will be observed that, as in the case of normal males, the mean output rises sharply with the onset of puberty and adolescence and reaches a maximum at about the age of twenty five Thereafter, according to Hamburger (1948), the curve for the 17 ketosteroid excretion in females is rather different from that in males Whereas in the latter a progressive fall occurs with advancing years the female subjects show a relatively sharp decrease in output between the ages of twenty five and thirty five fairly constant levels from thirty five to fifty five and a gradual fall in output after the age of sixty The urinary 17 ketosteroids in women arise almost exclusively from precursors secreted by the adrenal cortex, and it is reasonable to assume that the fluctuations at different ages represent at least to some extent variations in the secretory activity of this gland

A number of investigators including Werner (1941), Davis and Plotz (1956) and Borth *et al* (1957) have studied the 17 ketosteroid excretion throughout the normal menstrual cycle No definite cyclic fluctuations have been observed and the small fluctuations which do occur appear to bear no constant relationship to the various phases of the cycle

### 3 Normally Pregnant Women

Venning (1946) has estimated the 17 ketosteroid excretion throughout normal pregnancy Two colorimetric assay methods were used, one depending on the Holtorff Koch modification of the Zimmermann reaction and the other on the antimony trichloride reaction of Pincus The material giving a reaction in the modified Zimmermann procedure increased gradually as pregnancy advanced while the material active in the antimony trichloride reaction remained relatively constant throughout the gestation period These divergent results can probably be explained on a methodological basis The modified Zimmermann procedure is not entirely specific for urinary 17 ketosteroids (see p 292) and is affected to some extent by the presence of 20 ketosteroids in urine extracts It is therefore probable that the increased excretion of 5$\beta$ pregnan 3$\alpha$ ol 20 one and of other urinary 20 ketosteroids as pregnancy advances is sufficient to account for the apparent rise in 17 ketosteroid output as measured by the Holtorff Koch

The quantity of 17 ketosteroids excreted appears to depend to a large extent on the age at which the hyperplasia develops In female subjects in whom adrenal hypertrophy occurs prior to puberty the output of urinary 17 ketosteroids is generally abnormally high for the corresponding age period in healthy individuals, values lie between 30 and 100 mg per twenty four hours in most cases Such patients usually show features of the adrenogenital syndrome rather than of Cushing s syndrome On the other hand in females in whom the adrenal hypertrophy develops after puberty, the urinary 17 ketosteroid excretion tends to lie within or slightly above the normal range The mean value in 131 such cases collected from the literature by Mason and Engstrom (1950) was approximately 20 mg per twenty four hours These authors have, however wisely emphasised that many of these 131 patients had been classified by different workers under such headings as adrenal virilism simple hirsutism and post puberal virilism and that it is by no means certain that all the cases were true examples of adrenocortical hyperplasia

In recent years considerable attention has been given particularly by workers in the United States to the condition known as *congenital adrenal hyperplasia* This disease can occur in both sexes but is much more common in girls than in boys, in girls the condition has been termed female pseudo hermaphroditism In both sexes the hormonal abnormality occurs during embryonic life before intra uterine differentiation of the sex organs is complete The clinical features in females are generally those of the adrenogenital syndrome and have been fully described by numerous investigators including Wilkins (1950) and Bishop (1954) The excretion of 17 ketosteroids is almost always increased in relation to the patient s age During the first year of life, however the output may not exceed 2 to 3 mg per twenty four hours although levels as high as 15 mg per twenty four hours have been reported When congenital adrenal hyperplasia occurs in boys the clinical picture is generally one of precocious sexual development and this condition has been termed *macro genitosomia præcox* In such individuals also the excretion of urinary 17 ketosteroids is abnormally high in relation to the patient s age Values of 2 to 4 mg per twenty four hours have been reported in early childhood and levels of 20 to 50 mg

in such patients have been conducted by numerous investigators including Fraser *et al* (1941), Kepler and Mason (1947), Huis in t Veld and Dingemanse (1948), Walters and Sprague (1949) and Sobel *et al* (1953) In the majority of cases of proved adrenocortical tumour increased quantities of 17 ketosteroids are present in urine and in only a small proportion of subjects are the levels within the normal range Values of more than 1 000 mg per twenty four hours have been reported Forbes and Albright (1951) have stated that in cases of Cushing s syndrome there is a tendency for the 17 ketosteroid excretion to be relatively low when a benign tumour is responsible for the disease On the other hand there is generally but not always a massive excretion of urinary 17 ketosteroids when the tumour is malignant According to Mason and Engstrom (1950) a reading of over 150 mg per twenty four hours is diagnostic of an adrenocortical tumour in an adult female Such tumours are much less frequently encountered in adult males and in the few cases reported in the literature the output has been very variable In children the levels are also very variable, but in general are many times higher than those found in normal children at a corresponding age period

When separate estimation of the 3$\beta$ hydroxy fraction has been made in cases of adrenocortical neoplasm it has generally been found that this fraction is abnormally high, its proportion ranging from 30 to 90 per cent of the total 17 ketosteroid output as compared with less than 15 per cent in normal individuals Usually the main component of the 3$\beta$ hydroxy fraction has been shown to be dehydro*epi*androsterone It should, however, be emphasised that this elevation of the 3$\beta$ hydroxy fraction is not invariable and that occasionally a normal value may be found in the presence of a tumour

(*b*) Adrenocortical Hyperplasia —This is a very loose term which is used to denote the presence of bilaterally enlarged adrenal glands in the absence of neoplastic change As with adrenocortical tumour patients with hyperplasia generally present with the symptoms of Cushing s syndrome or of the adrenogenital syndrome Mixed types are also common Many workers including Escamilla (1949) and Seckel *et al* (1949) have studied the 17 ketosteroid output in patients with this disease

(c) ADDISON S DISEASE —The excretion of 17 ketosteroids in patients with this condition is usually abnormally low (Fraser *et al* 1941, Escamilla, 1949) Frequently readings of 1 mg per twenty four hours have been reported and, particularly in women the output may fall to zero According to Mason and Engstrom (1950) values of 1 mg or less in women or 3 mg or less in men are suggestive of the disease It should, however, be emphasised that such readings are by no means pathognomonic of Addison s disease and can be found in many other disease states On the other hand it can probably be stated with some assurance that the finding of urinary 17 ketosteroid levels which are within the normal range would militate against the diagnosis of Addison s disease especially in female patients

## 2 Diseases of the Pituitary

In *acromegaly* the excretion of urinary 17 ketosteroids is very variable (Fraser *et al* 1941, Venning and Browne, 1947, Escamilla, 1949) The levels may be within above or below the normal range In most cases reported in the literature the values have not exceeded 25 mg per twenty four hours and have not fallen below 5 mg per twenty four hours An increase in output has been observed more frequently in men than in women In cases of *pituitary gigantism* stigmata of hypogonadism are not uncommon and in such patients the 17 ketosteroid excretion is normal or slightly reduced

In *panhypopituitarism* the output of 17 ketosteroids is markedly decreased and levels below 1 mg per twenty four hours are usually encountered (Fraser *et al* 1941) Similar low levels are found in cases of *pituitary dwarfism*

## 3 Diseases of the Thyroid

The output of 17 ketosteroids is usually below the normal range in patients with untreated *myxœdema* (Fraser *et al* 1941 Engstrom and Mason 1944 Statland and Lerman 1950) Values may vary from zero to the lower limits of normal and are often less than 2 mg per twenty four hours Engstrom and Mason (1944) have shown that treatment of such patients with thyroid extract in a dose sufficient to relieve symptoms does not always cause a rise in the urinary excretion of 17 ketosteroids A similar conclusion was reached by Friedgood

per twenty four hours in late childhood Wilkins *et al* (1951) and others have shown that the oral or parenteral administration of cortisone to patients with congenital adrenal hyperplasia diminishes the excretion of urinary 17 ketosteroids and usually produces symptomatic improvement

THE ESTIMATION OF THE 3β HYDROXY FRACTION IN THE DIFFERENTIAL DIAGNOSIS OF PATIENTS WITH HYPERFUNCTIONAL ADRENAL DISEASE —As mentioned previously (p 293) separation of the 3α hydroxy and 3β hydroxy fractions can be effected by treatment of urine extracts with digitonin The principal constituent of the 3β hydroxy fraction is the substance dehydro*epi*androsterone In normal subjects the 3β hydroxy fraction is generally less than 15 per cent of the total 17 ketosteroid output and usually averages between 5 and 10 per cent (Dorfman and Shipley 1956)

In patients with adrenocortical tumour very high values for the 3β hydroxy fraction are almost always obtained (Callow *et al*, 1940, Hirschmann and Hirschmann, 1947, Kinsell and Lisser, 1952) In a series of twenty five cases collected from the literature by Dorfman and Shipley (1956) this fraction varied from zero to 79 per cent of the total 17 ketosteroid output with a mean value of 45 per cent, in only four out of the twenty five cases were the values within the normal range At present it cannot be stated with certainty whether there is any correlation between the excretory level of the 3β hydroxy fraction on the one hand and the degree of malignancy of the tumour on the other

The output of the 3β hydroxy fraction in patients with adrenocortical hyperplasia differs markedly from that found in cases of tumour although the excretion of total 17 ketosteroids may be abnormally high in both conditions Patients with hyperplastic adrenals excrete amounts of the fraction which are usually within the normal range or are only slightly elevated (Kepler and Mason, 1947 Dobriner *et al*, 1948) In a series of twenty nine cases collected from the literature by Dorfman and Shipley (1956) the 3β hydroxy fraction ranged from zero to 24 per cent of the total 17 ketosteroid output and the mean figure was 9 per cent It is therefore reasonable to conclude that the assay of this fraction may frequently be of value in differentiating adrenocortical tumour from adrenocortical hyperplasia

(c) Addison's Disease —The excretion of 17 ketosteroids in patients with this condition is usually abnormally low (Fraser *et al*, 1941, Escamilla, 1949) Frequently readings of 1 mg per twenty four hours have been reported and, particularly in women the output may fall to zero According to Mason and Engstrom (1950) values of 1 mg or less in women or 3 mg or less in men are suggestive of the disease It should, however, be emphasised that such readings are by no means pathognomonic of Addison s disease and can be found in many other disease states On the other hand it can probably be stated with some assurance that the finding of urinary 17 ketosteroid levels which are within the normal range would militate against the diagnosis of Addison s disease, especially in female patients

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(1944) According to Talbot and Butler (1942) hypothyroid children excrete smaller amounts of 17 ketosteroids than do normal children at a corresponding age period

In *thyrotoxicosis* 17 ketosteroid determinations in urine are of little diagnostic value As shown by Engstrom and Mason (1944), Kinnunen and Kauppinen (1951) and others the levels are usually within or slightly below the normal range

## 4 Diseases of the Testis

In the investigation of cases of male hypogonadism urinary 17 ketosteroid determinations have not so far proved of great diagnostic value to the clinician It should be borne in mind that the testes contribute only approximately one third of the total output of urinary 17 ketosteroids and that the remaining two thirds arise from precursors secreted by the adrenal cortex

In patients with *eunuchoidism* the urinary excretion of 17 ketosteroids is very variable In a relatively large series of cases collected from the literature by Dorfman and Shipley (1956) the output ranged from 1 4 to 15 7 mg per twenty four hours and the mean value was 6 6 mg

Numerous investigators including Scott and Vermeulen (1942) Dean *et al* (1944) and Birke *et al* (1954) have studied the 17 ketosteroid output after *castration* It has been generally found that the excretion falls immediately after operation but soon rises again to levels similar to or slightly higher than those observed pre operatively

In the rare condition described by Klinefelter *et al* (1942) and usually known as *sclerosing tubular degeneration* (Klinefelter s syndrome) low levels of urinary 17 ketosteroids have usually been reported

Relatively few 17 ketosteroid assays have so far been conducted in patients with *testicular tumours* In a case of *interstitial cell tumour* studied by Venning *et al* (1942) the output was 1 015 mg per twenty four hours, but in other patients with this condition reported by Cook *et al* (1952) and by Newns (1952) the excretion has been within the normal range In four patients with *seminoma* investigated by Warren (1945) the figures ranged from 15 4 to 32 2 mg per twenty four hours with a mean value of 21 4 mg In cases of *teratoma* the readings may be within or slightly above the normal range (Warren 1945, Dorfman and Shipley 1956)

## 5 Diseases of the Ovary

No consistent abnormality in 17 ketosteroid excretion has yet been demonstrated in cases of primary or secondary *amenorrhœa* or in patients with the various types of *dysfunctional uterine hæmorrhage*

Few assays have so far been reported in patients with virilising ovarian tumours such as *arrhenoblastoma* such readings as were obtained were very variable, lying within below or above the normal range (Pederson 1947 Riley and Murphy 1951) Most cases showed post operative values which were lower than those obtained pre operatively In *Turner s syndrome* (ovarian agenesis) a moderate depression of 17 ketosteroid output has been noted, in a series of eleven cases reported by Albright *et al* (1942) the levels lay between 2 and 5 mg per twenty four hours

In twenty nine patients with the *Stein Leventhal syndrome* collected from the literature by Dorfman and Shipley (1956) the 17 ketosteroid excretion was abnormally high in only five instances Most investigators have found that the output of 17 ketosteroids is not materially affected by *oophorectomy*

## 6 Malignant Disease

The excretion of 17 ketosteroids has been studied in patients with many forms of carcinoma These investigations have been prompted by the suggestion that steroid hormones may have some relationship to human carcinogenesis So far results obtained have been disappointing from the diagnostic point of view Although the total 17 ketosteroid excretion in malignant disease is often abnormally low it is difficult to assess whether this depression results from the cancer *per se* or from concomitant factors such as starvation and inanition Fractionation of the individual ketosteroids by chromatographic methods has been performed by a number of workers in various types of carcinoma The results obtained will be discussed later in this chapter

## 7 Miscellaneous Conditions

17 Ketosteroid assays have been conducted in a wide variety of other clinical conditions, a few of which are mentioned below

(*a*) ANOREXIA NERVOSA—Low values for urinary 17 ketosteroids are generally obtained, although occasionally the readings lie within the normal range (Fraser *et al*, 1941, Venning and Browne, 1947) Usually the output returns to normal with adequate nutrition 17 Ketosteroid assay *per se* is not a reliable method of differentiating cases of anorexia nervosa from those of panhypopituitarism

(*b*) HYPERTENSION—Bruger *et al* (1944) claimed that the excretion of 17 ketosteroids in hypertensive women was abnormally low However, other investigators have been unable to demonstrate any significant difference in 17 ketosteroid output between normotensive and hypertensive subjects

(*c*) LIVER DISEASE—There is general agreement that in cases of advanced liver disease the amounts of 17 ketosteroids found in urine are greatly decreased (Dohan *et al*, 1952, Warter *et al*, 1953) In a recent study involving twenty three cases of portal cirrhosis and five cases of infective hepatitis Birke (1954 *a*) found that the total excretion of 17 ketosteroids was depressed in sixteen cases while in the remaining twelve the values obtained were within the normal range The lowest levels of urinary 17 ketosteroids were found in those *patients with the greatest degree of impairment of liver function* All the normally occurring 17 ketosteroids were markedly reduced in advanced liver disease and no abnormal steroids were demonstrated

(*d*) RHEUMATOID ARTHRITIS—No consistent abnormality in 17 ketosteroid excretion has been demonstrated in patients with this disease (Desmarais, 1949, Sprechler, 1950) In a recent investigation involving forty cases Birke (1954 *b*) found that the majority of readings were within the normal range and only a few were abnormally low Fractionation studies by chromatographic methods combined with infra red spectroscopy failed to demonstrate the presence of any abnormal 17 ketosteroids in these subjects

(*e*) GOUT—Wolfson *et al* (1949) have claimed that in patients with gouty arthritis a consistently low excretion of 17 ketosteroids is found This interesting observation requires further confirmation

(*f*) SIMPLE HIRSUTISM—In this condition hirsutism occurs in the absence of demonstrable pathological lesions of the

adrenal cortex and the ovary The cause of the disease is at present unknown and none of the endocrine theories put forward to explain its clinical features are entirely satisfactory It should be emphasised that in many cases classified in the literature as simple hirsutism, adrenal or ovarian disease has not been definitely excluded by laparotomy

In most series of cases of simple hirsutism so far reported the mean 17 ketosteroid excretion has been significantly higher than that occurring in normal women of a comparable age group This has been shown by various investigators including Warren (1945) Patterson *et al* (1942) and Dorfman and Shipley (1956) There does not, however appear to be any definite correlation between the severity of the hirsutism on the one hand and the output of 17 ketosteroids on the other

(*g*) STRESS CONDITIONS —The 17 ketosteroid excretion is markedly affected by such factors as emotional stress and physical exertion Pincus (1947) has made extensive studies on the diurnal variation in urinary 17 ketosteroid excretion in normal subjects He found that the levels were lowest during sleep were at a maximum in the hour immediately after rising and gradually declined during the day This worker also demonstrated that conditions necessitating precise muscular co ordination such as operation of a pursuit meter resulted in a sharp rise in 17 ketosteroid excretion Pincus and Hoagland (1943) studied the effect of piloting aircraft on the 17 ketosteroid excretion they found that this stress produced a marked rise in output and that the increase was directly proportional to the time spent in the air

Forbes *et al* (1947) have investigated the effect of major surgery on the output of urinary 17 ketosteroids They found that some twenty four or forty eight hours after the operation the excretion rose above normal limits Subsequently a gradual fall in output occurred and some four to five days post operatively the readings were frequently below the normal range Within ten days of the operation the values had returned to normal Eisenmann *et al* (1953) have reported marked alterations in 17 ketosteroid excretion during the withdrawal of morphine from addicted patients During the time of addiction the excretion of these steroids was abnormally low following withdrawal of the drug the excretion rose sharply to levels above the normal range

For a fuller description of the relationship of stress conditions to 17 ketosteroid output in normal and disease states the reader is referred to articles by Pincus (1947), Sayers (1950) and Mason and Engstrom (1950)

## THE URINARY EXCRETION OF THE INDIVIDUAL 17-KETOSTEROIDS IN NORMAL AND PATHOLOGICAL CONDITIONS

### 1 Normal Subjects

The excretion of the individual urinary 17 ketosteroids by normal individuals has been investigated by Dingemanse *et al* (1952) Pond (1954) Plantin and Birke (1954), Rubin *et al*

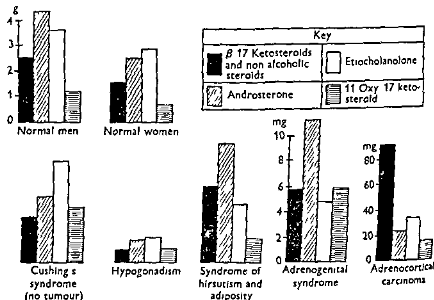



FIG 64

A graphic summary of the average 17 ketosteroid fractionation results in various endocrine disorders (*From Pond* 1954)

(1954) and Johnsen (1956 *b*) In a careful study involving twenty normal subjects Pond (1954) found that androsterone and $5\beta$ androstanolone constituted approximately two thirds of the total 17 ketosteroid excretion The ratio of the two compounds was approximately unity in both sexes The levels of 11 oxy 17 ketosteroids remained relatively constant from

day to day but were appreciably higher in men than in women The 3$\beta$ hydroxy fraction usually constituted approximately 20 per cent of the total 17 ketosteroid output This figure is rather higher than that obtained by other workers A summary of Pond s results in normal and pathological conditions is shown in Fig 64

**2 Abnormal Conditions**

At the time of writing relatively few systematic investigations have been undertaken of the excretion of the individual 17 ketosteroids in pathological conditions in man It is therefore too early to assess the possible value of such assays in clinical medicine It is, however, probable that studies of this type will in the future yield information of considerable clinical importance Some of the preliminary results obtained in various disease states are discussed below

(*a*) Cushing s Syndrome —In nine cases of this disease studied by Pond (1954) the main change in the urinary 17 ketosteroid pattern consisted of an increased excretion of 11 oxy 17 ketosteroids In the majority of patients the ratio 5$\alpha$ androstanolone 5$\beta$ androstanolone was less than unity and the 3$\beta$ hydroxy fraction exceeded 50 per cent of the total 17 ketosteroid output

(*b*) Adrenogenital Syndrome —In patients with this condition Pond (1954) found that the excretion of the individual 17 ketosteroids was different from that in Cushing s syndrome The ratio 5$\alpha$ androstanolone 5$\beta$ androstanolone was generally greater than unity and the excretion of the 11 oxy 17 ketosteroids was not abnormally high

(*c*) Male Hypogonadism —In four cases Pond (1954) observed a lowered excretion of all 17 ketosteroid fractions, the relative rise in 3$\beta$ hydroxy 17 ketosteroids noted by Callow *et al* (1940) was not demonstrated In a study of twelve male castrates Johnsen (1956 *c*) reported an abnormally low total 17 ketosteroid excretion a relative decrease in androsterone output and a relative increase in the excretion of the 3$\beta$ hydroxy 17 ketosteroids particularly dehydro*epi*androsterone In Johnsen s opinion the estimation of the individual 17 ketosteroids in urine is of greater diagnostic value in the investigation of patients with male hypogonadism than is the determination of the total 17 ketosteroid excretion

(*d*) Diseases of the Pituitary —In four cases of panhypopituitarism Pond (1954) found a reduction in all 17 ketosteroid fractions Johnsen (1956 *c*) has demonstrated that, after hypophysectomy performed for recurrent mammary carcinoma, there is a marked lowering of all fractions but there is in addition a relative decrease in androsterone excretion This interesting finding requires further investigation

(*e*) Liver Disease —Recently Birke (1954 *a*) has studied the excretion of the individual 17 *ketosteroids in patients* with portal cirrhosis and with infective hepatitis (see p 306) He found that all the principal metabolites in urine, viz androsterone, dehydro*epi*androsterone $5\beta$ androstanolone and 11 ketoætiocholanolone were markedly decreased in a proportion of cases However, it was not possible to demonstrate the presence of any pathological steroid in the urine of any of the patients investigated Results similar to those reported by Birke (1954 *a*) have been obtained by Marti (1951)

(*f*) Rheumatoid Arthritis —In a study of forty cases Birke (1954 *b*) found no alteration in the pattern of 17 ketosteroid excretion as compared with healthy subjects All the principal metabolites occurring in normal urine were identified by means of infra red spectroscopy and no pathological steroids were demonstrated

(*g*) Malignant Disease —Various investigators have fractionated the individual urinary 17 ketosteroids in patients with different types of carcinoma Although deviations from the normal pattern have been observed it would be premature at the present time to draw any definite conclusions *regarding* the diagnostic value of such estimations

In 1947 Dobriner *et al* stated that the urine of patients with various types of malignant disease contained the steroid $3\alpha$ hydroxy $5\alpha$ androst 9 en 17 one This substance is probably produced from $11\beta$ hydroxyætiocholanolone by dehydration during the acid hydrolysis of urine The presence of this steroid was not detected in the urine of normal individuals, and since this original work of Dobriner and his co workers it has been generally assumed that 11 hydroxy ætiocholanolone has some connection with malignant disease The original claims have not been confirmed by subsequent investigators who used more reliable assay methods than those employed by Dobriner *et al* (1947) For example, Plantin

and Birke (1955) showed that although the urine of patients with prostatic carcinoma contained relatively large quantities of 11β hydroxyætiocholanolone the steroid was also present in easily detectable amounts in the urine of normal subjects and in samples obtained from patients with rheumatoid arthritis and portal cirrhosis In view of these observations it can be stated with assurance that the presence of this compound in urine is in no way pathognomonic of the existence of neoplastic disease

Birke *et al* (1954) have recently studied the pattern of 17 ketosteroid excretion in patients with carcinoma of the prostate They found that such cases prior to therapy usually excreted relatively large quantities of the steroid 11 ketoætiocholanolone but that the output of the androgenic steroids androsterone and 5β androstanolone was not abnormally high Orchidectomy produced a marked fall in the excretion of both androsterone and 5β androstanolone but within a few weeks the output of those steroids rose again to levels similar to or slightly higher than those observed pre operatively In patients who had been subjected to both orchidectomy and adrenalectomy the excretion of androsterone and 5β androstanolone fell to zero

(*h*) SURGICAL STRESS —Birke *et al* (1955) have recently reported the effect of various types of major surgery on the excretion of the individual urinary 17 ketosteroids The most striking features observed in the immediate post operative period was a sharp rise in the excretion of dehydro*epi*androsterone to levels approximately 200 per cent higher than those encountered prior to surgery The excretions of androsterone and 5β androstanolone also increased post operatively but to a smaller extent the output of 11 oxy 17 ketosteroids was not immediately affected by the operation Within nine to eleven days after surgery the excretion of all 17 ketosteroid fractions had returned to normal The level of dehydro*epi*androsterone in urine fell rapidly and was within the normal range after seven to nine days androsterone and 5β androstanolone had returned to pre operative levels within three days The 11 oxy 17 ketosteroids showed a slight increase in excretion on the third or fourth post operative day but were again within normal limits approximately ten days after operation

## II NEUTRAL 17-KETOSTEROIDS IN BLOOD

### 1 Methods of Estimation

Gardner (1953) has described a method for the estimation of 17 ketosteroids in plasma The compounds are liberated from the plasma proteins by acid hydrolysis and the extracts are purified by column chromatography using Florosil The final colorimetric determination depends on a micro modification of the Zimmermann reaction Results are expressed in terms of dehydro*epi*androsterone A similar technique in which the extracts were purified by both column and paper chromatography was subsequently described by Migeon and Plager (1955) These latter workers claimed to have isolated two fractions from plasma, one containing dehydro*epi*androsterone and the other a mixture of androsterone and 5$\beta$ androstanolone

It should be emphasised that the techniques so far developed for the estimation of 17 ketosteroids in blood, although promising, are not yet entirely satisfactory from the quantitative point of view Further methodological work is required before these procedures can be used routinely in clinical studies

### 2 Assays in Clinical Conditions

Migeon (1954) has recently reported some preliminary results on the plasma 17 ketosteroid concentration in health and disease In normal subjects of both sexes the two fractions mentioned above could be detected The intravenous administration of ACTH to normal subjects caused a rise in both fractions but the increase in dehydro*epi*androsterone concentration was more marked than that of androsterone and 5$\beta$ androstanolone In two patients with Cushing s syndrome resulting from adrenocortical carcinoma very high blood concentrations of dehydro*epi*androsterone were found Abnormally high titres of this steroid were also reported in cases of congenital adrenal hyperplasia in children

## III ANDROGENS IN BLOOD AND URINE

The term androgen will be used to denote the group of compounds present in extracts of blood and urine which possess biological activity It has already been emphasised (p 290)

that the various urinary 17 ketosteroids differ markedly in their androgenic potencies and that the terms androgen and '17 ketosteroid are by no means synonymous

## METHODS OF ASSAY OF THE ANDROGENS

Results of androgen bio assays should always be expressed in terms of the international standard preparation and not in animal units By definition one international unit represents the androgenic activity of 0 1 mg of pure androsterone The subject of androgen assay has recently been thoroughly reviewed by Dorfman and his co workers (Dorfman 1950 Dorfman and Shipley, 1956) and the present account is based mainly on their conclusions

Bio assay methods can be conveniently divided into two main groups These are

1 Assays in birds
2 Assays in mammals

### 1 Assays in Birds

(a) THE CAPON S COMB TEST —This has been the most widely used method for assessing androgenic potency It depends on the fact that after castration marked involution of the cock s comb occurs and that the administration of androgens to these birds restores the comb to its normal size The birds are ready for use some six months after castration In designing the assay most investigators have employed either a three day or a five day experimental period The material under test can be given by intramuscular injection or by direct application to the comb The former route has been used by various workers including Gallagher and Koch (1935) Greenwood *et al* (1935) and Emmens (1939) while the latter has been recommended by McCullagh and Cuyler (1936) Deanesly and Parkes (1937) and Emmens (1939) Assays in which androgens are administered by inunction are many times more sensitive than those in which the intramuscular route is used

The capon s comb test has been the most popular bio assay technique for estimating the concentration of androgens in human blood and urine The results obtained by its use will be discussed later in this chapter

(*b*) Chick Comb Methods—Such methods have been described by Klempner *et al* (1942), Frank *et al* (1942), Valle *et al* (1947) and Dorfman (1950) Different breeds of chicks vary greatly in their sensitivity to androgens, and it has been shown that both male and female white Leghorns are many times more sensitive than Rhode Island Reds or Barred Rocks *In the assay method described by Frank et al* (1942) white Leghorn chicks of either sex at two or three days

TABLE XVIII

Precision of Assay Methods for Androgens in Birds

(*After Dorfman and Shipley* 1956)

| Method | Mode of Administration of Androgens | Index of Precision λ | Reference |
|---|---|---|---|
| Capon s comb | Intramuscular | 0 189 | Greenwood *et al* (1935) |
| Capon s comb | Intramuscular | 0 271 | McCullagh and Cuyler (1936) |
| Capon s comb | Inunction | 0 184 | Emmens (1939) |
| Capon s comb | Inunction | 0 076 | McCullagh and Cuyler (1936) |
| Chick s comb | Inunction | 0 424 | Valle *et al* (1947) |
| Chick s comb | Inunction | 0 334 | Dorfman (1950) |

*of age are used The solutions under test are administered by* inunction and the experimental period is seven days The birds are killed twenty four hours after the last application of the androgen solutions and the end point of the assay depends on comb weight

In general assays involving chicks are more sensitive more convenient but less precise than those employing capons In *Table XVIII, which is based on data calculated by Dorfman* and Shipley (1956), the precision of the various assay methods in birds is compared in terms of Gaddum s λ

## 2 Assays in Mammals

The most popular assay *methods in mammals* have depended on the enlargement of the prostate of the seminal

vesicles or of the total accessory reproductive organs in castrated rats (Korenchevsky *et al* 1935 Callow and Deanesly, 1935, Bulbring and Burn 1935) Such assays are less sensitive than those employing birds and are not suitable for use in clinical studies For details of the actual techniques recommended the reader is referred to the original articles

## THE CLINICAL APPLICATION OF ANDROGEN ASSAYS

Bio assay methods for androgens are time consuming and unreliable while chemical methods for the determination of 17 ketosteroids are much more precise much less laborious and therefore much more suited for routine clinical use Accordingly considerably more data is available in the literature on the urinary 17 ketosteroid excretion in health and disease than on the androgen excretion under similar conditions

It should not be assumed however that the information obtained by 17 ketosteroid assays on the one hand and by androgen assays on the other is identical This point has been stressed by Hamburger (1948) and by Hamilton (1954) Hamburger (1948) showed that castration in men caused a much greater fall in the excretion of biologically active androgens than in the excretion of 17 ketosteroids and concluded that under these circumstances biological androgen determinations gave a better index of testicular function than did urinary 17 ketosteroid estimations Hamilton (1954) has recently studied the relationship of androgen excretion to 17 ketosteroid excretion in normal and disease states He expressed his results in the form of an index calculated as the androgenic activity per milligramme of colorimetrically determined 17 ketosteroid He found that the index differed markedly with the age sex and clinical condition of the individuals The index was lower in women than in men and was particularly low in male castrates The explanation of these findings lies in the fact that the various urinary 17 ketosteroids vary greatly in their androgenic activities Accordingly the androgenic potency of a given urine extract will not necessarily parallel the 17 ketosteroid concentration

In the opinion of Hamburger (1948) the chemical determination of 17 ketosteroids in urine has not rendered the old

biological assays superfluous It is, however, unlikely that such assays in view of their many disadvantages from the practical standpoint, will ever again find favour in clinical investigations For this reason only a brief account of the androgen excretion in health and disease will be given below Most of the data presented has been summarised from a recent publication by Dorfman and Shipley (1956)

**1 The Urinary Excretion of Androgens in Normal Subjects**

In normal *men* the excretion of androgens has been found to be very variable and the mean values in different laboratories have ranged from 20 to 100 i u per twenty four hours In normal non pregnant *women* androgen excretion has not been shown to bear any constant relationship to the time of the cycle and the mean figures in different centres have varied from 15 to 50 i u per twenty four hours In *children* androgens can be detected in the urine in very small amounts at the age of two or three From the age of seven or eight until puberty a gradual increase in output is noted in both boys and girls, no significant difference between the sexes is evident prior to puberty Normal adult levels are not generally attained until about the age of seventeen to twenty In *old men* and *old women* the output of urinary androgens diminishes with advancing years In a careful study Hamburger *et al* (1945) found that men between the ages of fifty and sixty excreted quantities of androgens which were only slightly smaller than those in young men In the sixty year to seventy year age group, however, the mean output was approximately one third of that in young men while in subjects between seventy and eighty the figure had dropped to one quarter of that found in early adult life

For further information on the subject of androgen excretion in normal subjects the reader is referred to articles by Gallagher *et al* (1937), Dingemanse *et al* (1937), Dorfman *et al* (1937) and Hamburger *et al* (1945)

**2 The Urinary Excretion of Androgens in Pathological Conditions**

Information on this subject in the literature is scanty and is generally unsuitable for statistical evaluation

In patients with diseases of the *adrenal cortex* the androgen

excretion follows the same general trends as those already described for the 17 ketosteroids Abnormally high values are usually encountered in cases of adrenocortical tumour and the readings tend to be especially high if the tumour is malignant Raised excretion values have been reported in some but not all cases of adrenocortical hyperplasia In Addison s disease on the other hand, most investigators have found a well marked fall in androgen excretion

There has been some interest in the androgen output in patients with *testicular disease* In eunuchoidism the excretion is very variable but is usually lower than in healthy individuals of a comparable age group After castration androgen continues to be excreted but the quantities found in urine are very small Hamburger (1948) has pointed out that castration in man causes a much greater fall in the androgen excretion than in the output of urinary 17 ketosteroids

### 3 Androgen Estimations in Blood under Normal and Pathological Conditions

The most careful study of the androgen concentration of human blood so far reported is that of Tornblom (1946) This worker assayed the neutral fraction obtained from blood by the capon s comb test and expressed his results in terms of testosterone In normal *men* between the ages of twenty and thirty the mean concentration was $4\ 96 \pm 0\ 59$[1] $\mu g$ per 100 ml of blood The androgen content of blood decreased with advancing years and between the ages of fifty and sixty was approximately one third of that found in young men In normal *women* from twenty to forty years of age the mean concentration was $7\ 5 \pm 3\ 5$[1] $\mu g$ per 100 ml In castrated men and oophorectomised women abnormally low blood androgen titres were found In a small series of hirsute women the readings were within the normal range

## Summary and Conclusions

Total urinary 17 ketosteroids can be estimated either by colorimetric or by polarographic methods Most workers employ the Callow Zimmermann technique or some modification thereof Recently a number of micro methods

[1] Standard error of the mean

depending on the Zimmermann reaction have been developed and some of these are suitable for routine use Techniques for the estimation of neutral 17 ketosteroids in blood are not yet sufficiently reliable to allow of their widespread use in clinical studies

Fractionation of the individual urinary 17 ketosteroids can be performed by chromatographic methods Such procedures may in the future yield information of considerable diagnostic value

In normal individuals of both sexes the 17 ketosteroid levels in urine rise rapidly prior to puberty, reach a maximum at about the age of twenty five and fall progressively with advancing years In normal subjects the 3$\beta$ hydroxy fraction is usually less than 15 per cent of the total 17 ketosteroid output

Abnormally high 17 ketosteroid excretion values are generally found in patients with adrenocortical tumours and the levels are particularly high if the tumour is malignant In such cases the 3$\beta$ hydroxy fraction often constitutes over 40 per cent of the total 17 ketosteroid output In adrenocortical hyperplasia, on the other hand high levels are frequently but by no means invariably encountered and in such patients the 3$\beta$ hydroxy fraction is generally within normal limits In Addison s disease the 17 ketosteroid excretion is below the normal range in most cases

An abnormally low excretion of total urinary 17 ketosteroids is usually found in panhypopituitarism in anorexia nervosa, in myxœdema and in advanced liver disease Raised excretion values have been reported in stress conditions, in simple hirsutism in interstitial cell tumour of the testis and in arrhenoblastoma

Androgen assays in blood and urine can be conducted by the capon s comb test or by one or other of the chick comb methods Such assays are not very satisfactory from the quantitative point of view, and it is therefore unlikely that they will continue to be used in clinical investigations

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## CHAPTER XIII

# *Adrenaline and Noradrenaline*

## INTRODUCTION

BOTH adrenaline and noradrenaline are produced by the adrenal medulla while noradrenaline is now recognised as the main substance liberated by post ganglionic sympathetic nerves Another name for adrenaline is *epinephrine* while noradrenaline is sometimes referred to as *norepinephrine* or *arterenol*

### 1 Chemical Nature of Adrenaline and Noradrenaline

The structural formulæ of adrenaline and noradrenaline are shown in Fig 65

It will be noted that the two compounds resemble one

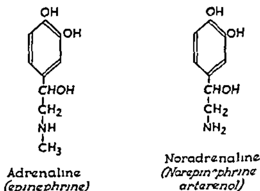



Fig 65
Structural formulæ of adrenaline and noradrenaline

another very closely in chemical structure The only difference is that in noradrenaline the nitrogen in the terminal amino ($NH_2$) group is not methylated Noradrenaline is a primary amine while adrenaline is a secondary amine Both compounds are generally referred to as *catecholamines* The lævo isomer of adrenaline (*l* adrenaline) occurs naturally in the

adrenal gland and has much greater pharmacological activity than the dextro isomer Tullar (1948) succeeded in resolving racemic noradrenaline into its lævo and dextro isomers, and Luduena *et al* (1949) were able to show that in the case of this compound also the pharmacological activity resided mainly in the lævo fraction Adrenaline is stable in weak acids but is rapidly oxidised in neutral and alkaline solutions Noradrenaline is much more resistant to oxidation than adrenaline

## 2 Occurrence of Adrenaline and Noradrenaline in Body Fluids

The quantity of catecholamines present in *human peripheral blood* under physiological conditions is very small and frequently no activity can be detected even when very sensitive bio assay methods are used Little is at present known regarding the form in which adrenaline and noradrenaline circulate in blood Gaddum *et al* (1949) have emphasised that reliable estimations of catecholamines in blood are possible only if the blood is collected very carefully into a siliconed needle and a syringe containing heparin These precautions are designed to prevent the liberation from platelets of substances such as histamine and 5 hydroxytryptamine which may interfere with the bio assay

Adrenaline and noradrenaline are excreted in *urine* partly in the *free* form and partly *conjugated* with glucuronic acid (Holtz *et al* 1947 Kroneberg and Schumann 1950 Euler and Hellner 1951 Burn 1953) Euler and Luft (1951) found that when noradrenaline was administered to healthy subjects by intravenous infusion 2 to 3 per cent of the injected dose appeared in the urine as free noradrenaline but the adrenaline content of urine did not rise

The human adrenal medulla is believed to contain a mixture consisting of approximately 85 per cent of adrenaline and 15 per cent of noradrenaline (Euler 1955) In normal urine on the other hand the quantity of noradrenaline generally exceeds that of adrenaline This finding suggests that under normal conditions the large proportion of catecholamines in urine is derived from adrenergic nerves rather than from the adrenal medulla The excretion of adrenaline and nor adrenaline in human urine under normal and pathological con ditions in man will be considered in detail later in this chapter

## METHODS OF ESTIMATION OF ADRENALINE AND NORADRENALINE

A large number of methods have been proposed for the quantitative determination of catecholamines in blood and urine The subject has been recently reviewed by many investigators including Gaddum (1950), Euler (1950, 1955), Pekkarinen (1954), Robson and Keele (1956) and Gaddum and Holzbauer (1957) Table XIX which is taken from a paper

### TABLE XIX

SENSITIVITY OF VARIOUS ASSAY METHODS FOR ADRENALINE AND NORADRENALINE

*(After Gaddum and Holzbauer 1957)*

| Method of Assay | Amount (Nanogram ) required for each Test | |
|---|---|---|
| | Adrenaline | Noradrenaline |
| *Biological*— | | |
| Cat s blood pressure | 200 | 100 |
| Rat s blood pressure | 50 | 5 |
| Rat s blood pressure (pithed) | 7 | 5 |
| Rat s uterus (2 ml bath) | 0 1 | 15 |
| Rabbit s ear (perfused) | 0 5 | 1 |
| Rabbit s ear (Armin and Grant 1953) | 0 002 | |
| Rabbit s gut (10 ml bath) | 40 | 40 |
| Hen rectal cæcum (2 ml bath) | | 50 |
| *Chemical*— | | |
| Formation of adrenochrome | 10 000 | 10 000 |
| Reduction of arsenomolybdate | 50 | 800 |
| Formation of adrenolutin | 20 | 20 |
| Coupling with ethylenediamine | 6 | 6 |

The amounts required for a reasonably precise bio-assay would be five to ten times the amounts given above

Nanogram (ng ) = one thousandth of a microgram

by Gaddum and Holzbauer (1957) shows the approximate sensitivities of some of the procedures suggested

One of the main difficulties encountered in such estimations arises from the fact that body fluids contain very small quantities of catecholamines in association with other pharmacologically active substances such as histamine acetylcholine and 5 hydroxytryptamine which are liable to interfere with

the final determination Accordingly it is necessary to employ preliminary purification methods which remove interfering substances from extracts and which concentrate the catechola mines in these extracts Special procedures are also required to distinguish adrenaline from noradrenaline

The ensuing discussion will be conducted under four main headings These are

1 Methods of purification of extracts
2 Bio assay methods for adrenaline and noradrenaline
3 Chemical assay methods for adrenaline and nor adrenaline
4 Assays of mixtures of adrenaline and noradrenaline

## 1 Methods of Purification of Extracts

Such procedures have generally depended on chromato graphy either on columns or on paper Aluminium hydroxide and aluminium oxide have been much used as adsorbents These substances show a high degree of specificity for catechol derivatives which are adsorbed easily and completely In the method described by Shaw (1938) freshly precipitated aluminium hydroxide is shaken with a solution containing adrenaline at *p*H 4 and is then separated by centrifugation This removes some interfering substances The supernatant fluid is then adjusted to *p*H 8 5 and is shaken again with aluminium hydroxide which adsorbs adrenaline completely at this *p*H but leaves other substances behind in solution the adrenaline can then be recovered by dissolving the aluminium hydroxide in acid Euler (1948) slightly modified Shaw s method by precipitating the aluminium hydroxide in the presence of adrenaline and then precipitating the salts from acid solution by a mixture of acetone and alcohol In this way he obtained a solution which was suitable for bio-assay In more recent methods such as those described by Lund (1950) Crawford and Outschoorn (1951) and Weil Malherbe and Bone (1952) aluminium oxide is used as an adsorbent in place of aluminium hydroxide

Lecomte and Fischer (1949) passed their extracts through a column of Decalco which adsorbs both adrenaline and noradrenaline A solution of iodine at *p*H 4 is then used to convert adrenaline to adrenochrome and to elute it from the column Noradrenaline can be eluted at *p*H 7 Bergström

and Hansson (1951) have reported that the catecholamines can be successfully adsorbed on the ion exchange resin IRC 50

In recent years *paper chromatographic methods* have become increasingly popular for the purification of extracts containing adrenaline and noradrenaline (James 1948, Crawford and Outschoorn 1951, Vogt, 1954) After chromatographic separation the catecholamines can be eluted from the appropriate part of the paper and can then be estimated by bio assay

**2 Bio assay Methods for Adrenaline and Noradrenaline**

Many tests have been devised by means of which parallel assays can be performed on at least two preparations, one of which is more sensitive to adrenaline and the other to nor adrenaline In this way it is possible to estimate the total concentration of catecholamines present and to calculate in addition the relative amounts of adrenaline and noradrenaline According to Euler (1955) a satisfactory differentiation is usually obtained when the difference in ratios is tenfold or more The following techniques will be considered —

(*a*) BLOOD PRESSURE IN CATS AND RATS —The pressor effect in the spinal cat has been much used for the assay of cate cholamines in body fluids tissues and commercial extracts (Cannon and Rosenblueth 1933 Greer *et al*, 1938, Burn *et al*, 1950) This test is more sensitive to noradrenaline than to adrenaline (Table XIX)

Crawford and Outschoorn (1951) have shown that the pressor test in urethanised rats is especially sensitive to nor adrenaline and is much less sensitive to adrenaline Vogt (1952) found that the sensitivity of the preparation can be increased by the intravenous injection of hexamethonium ($C_6$) In this way as little as 15 ng of noradrenaline can be detected

(*b*) RAT S UTERUS —Although it had been known for many years that the isolated rat uterus is readily inhibited by adrenaline the assay was not put on a quantitative basis until the work of de Jalon *et al* (1945) who studied the many variable factors affecting the test Gaddum *et al* (1949) compared the rat uterus test with other assay methods and found that it was much more sensitive to adrenaline than to noradrenaline (Table XIX) These studies were extended by

Gaddum and Lembeck (1949) who concluded that nor adrenaline is unlikely to interfere with the use of this preparation for the assay of adrenaline unless the former compound is present in a very high concentration Under optimal conditions as little as 0 1 ng of adrenaline can be determined by this method

(c) RABBIT S EAR —Armin and Grant (1953) have described an assay method which will detect as little as 0 002 ng of adrenaline The drug is injected into the main artery of the rabbit s ear *in situ*, the preparation having been sensitised by degenerative section of the nerves The end point of the test consists in measuring the diameter of the artery at intervals with a microscope The technique is sufficiently sensitive to detect the presence of adrenaline in blood

(d) INTESTINAL MUSCLE IN RABBITS, RATS AND HENS — Catecholamines usually produce an inhibitory effect on intestinal muscle Rabbit s intestine is equally sensitive to adrenaline and noradrenaline (Table XIX) Rat s colon is especially sensitive to noradrenaline (Gaddum *et al*, 1949) but also responds to 5 hydroxytryptamine Hen s rectal cæcum is much more sensitive to adrenaline than to noradrenaline (Euler, 1948)

### 3 Chemical Assay Methods for Adrenaline and Nor adrenaline

Chemical estimations of catecholamines can be performed either by *colorimetric* or by *fluorimetric* methods

(a) COLORIMETRIC METHODS —Techniques depending on colorimetry have been described by Euler and Hamberg (1949) Suzuki and Ozaki (1951) and others Adrenaline is easily oxidised under suitable conditions to form a pink substance adrenochrome, and other pink indole compounds Various oxidising agents can be used including iodine (Euler and Hamberg 1949) and potassium permanganate (Suzuki and Ozaki, 1951) Such tests are relatively insensitive but are reasonably specific

Another group of colorimetric methods depends on the fact that adrenaline can act as a reducing agent In general such procedures are more sensitive but less specific than those mentioned above In the methods of Whitehorn (1935) and Shaw (1938) adrenaline reduces arsenomolybdic acid to a

blue compound The blue colour produced by adrenaline can be increased three to five times by preliminary treatment with alkali No such increase occurs in the case of noradrenaline and therefore this technique may be of value in differentiating between the two catecholamines

(*b*) FLUORIMETRIC METHODS —In 1930 Paget showed that adrenaline became fluorescent in alkaline solution Gaddum and Schild (1934) proposed an assay method based on this fact These workers noted that the fluorescence produced by noradrenaline was only 2 per cent of that of adrenaline and that other closely related substances were also comparatively inactive Ehrlen (1948) and Fischer (1949) have subsequently shown that the fluorescent material formed from adrenaline is adrenolutin

Lund (1949 *a*, *b*, *c*) has described a fluorimetric method for the quantitative determination of adrenaline and noradrenaline In this technique the catecholamines are oxidised to adreno chrome and noradrenochrome by means of manganese dioxide and are then converted to adrenolutin and noradrenolutin in the presence of alkali and ascorbic acid Adrenolutin and noradrenolutin give a strong yellowish green fluorescence in ultra violet light and this can be estimated in a sensitive fluorimeter Euler and Floding (1955) have described a similar method in which potassium ferricyanide acts as the oxidising agent instead of manganese dioxide The originators claim that this technique can measure as little as 20 ng of both adrenaline and noradrenaline

Weil Malherbe and Bone (1952) have described a different type of test in which the catechols are first oxidised to chromes and are then allowed to form fluorescent condensation products with ethylene diamine This method is very sensitive and will detect as little as 6 ng of adrenaline and noradrenaline (Table XIX) It is, however, relatively non specific as shown by the fact that catechol itself and various catechol derivatives are also capable of forming fluorescent compounds

In general it may be said that chemical methods are more convenient but less specific than bio assays It is probable however, that in the future improved chemical methods depending on fluorimetry will largely replace biological assays for the quantitative determination of catecholamines in body fluids

## 4 Assays of Mixtures of Adrenaline and Noradrenaline

This subject has been reviewed by Gaddum (1950) Mann and West (1950) Euler (1955) and Gaddum and Holzbauer (1957) Both biological and chemical methods can be used to calculate the relative proportions of adrenaline and noradrenaline in a mixture The technique generally employed is as follows —

Standard solutions of adrenaline and noradrenaline are compared with the unknown mixture by two different methods one of which is particularly sensitive to adrenaline and the other to noradrenaline The concentrations of the two catecholamines in the mixture can then be determined by appropriate mathematical methods

This method of calculation has been applied to various combinations of bio-assays Euler (1948) used the cat s blood pressure and the hen s rectal cæcum, Bulbring (1949) the rat s uterus and the cat s blood pressure Gaddum and Lembeck (1949) the rat s uterus and the rat s colon and Burn *et al* (1950) the cat s blood pressure and nictitating membrane Results calculated in this way are subject to large errors

A similar procedure can be adopted in the case of colorimetric or fluorimetric methods of assay (Euler and Hamberg 1949 Euler and Floding 1955) Under acid conditions adrenaline is more rapidly oxidised than noradrenaline and an estimate of the relative proportion of the two compounds can therefore be obtained by oxidising the mixture for appropriate times at two different *p*H s In the method described by Weil Malherbe and Bone (1952) the fluorescent light is exposed to filters of different colours Using a yellow filter the intensity of the fluorescent light from adrenaline is approximately four times stronger than that from noradrenaline when a blue green filter is employed the intensities are equal

If more accurate estimations are required the two substances should be separated by paper chromatographic methods prior to the final determination Holzbauer and Vogt (1954) have found that under these circumstances a very satisfactory combination of tests is the rat s uterus and the rat s blood pressure Using these methods it is possible to estimate as little as 0 5 ng of adrenaline and 15 ng of noradrenaline in a mixture

## THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE IN NORMAL SUBJECTS

The clinical applications of the assay of catecholamines in urine have been intensively studied by Euler and his collaborators in Stockholm and the present account is based mainly on their conclusions These workers purified their extracts by adsorbing the catecholamines on aluminium hydroxide The *final determination was made by bio assay* using the cat s blood pressure and the hen s rectal cæcum The excretion figures quoted are for the *free* compounds According to Euler (1955) the relationship between the free and total catecholamines in urine is rather variable, but

TABLE XX

URINARY EXCRETION OF FREE ADRENALINE AND NORADRENALINE IN HEALTHY SUBJECTS

(*After Euler* 1955)

| Condition | Adrenaline μg per Twenty four Hours | Noradrenaline μg per Twenty four Hours |
|---|---|---|
| Staying in bed | 2 to 4 | 10 to [illegible]0 |
| Resting (not in bed during day) | 3 to 6 | 20 to 40 |
| Moderate activity during day | 5 to 10 | 30 to 60 |

usually the figures for total catechols are one and a half to three times higher than those for the free compounds The difference can be explained by the presence in urine extracts of conjugated forms of adrenaline and noradrenaline (see p 323) Most investigators now believe that the noradrenaline in urine is derived mainly from the adrenergic nerves while the adrenaline comes predominantly from the adrenal medulla

In healthy subjects under physiological conditions the urinary excretion of adrenaline ranges from 1 5 to 8 μg per twenty four hours while for noradrenaline the corresponding figures are 15 to 20 μg per twenty four hours (Euler *et al*, 1955 *a*) Readings tend to be higher during the day than by night Table XX which is taken from a recent publication by Euler

(1955) shows the excretion of adrenaline and noradrenaline in healthy subjects at different times of the day and under varying conditions

In a recent study Elmadjian *et al* (1956 *b*) have reported that in healthy subjects the excretion of both noradrenaline and adrenaline is higher in the waking state than when asleep These workers state that the percentage increase of adrenaline under these circumstances is higher than that for noradrenaline

## FACTORS INFLUENCING THE EXCRETION OF ADRENALINE AND NORADRENALINE IN HEALTH AND DISEASE

### 1 Vascular Reflexes

Euler *et al* (1955 *b*) showed that healthy young subjects excrete much larger quantities of both adrenaline and nor adrenaline when tilted to +75 degrees than when recumbent It is probable that the increased excretion of noradrenaline *results* from the activation of the homeostatic *reflexes* which normally control blood pressure while the increased output of adrenaline probably reflects the moderate degree of stress involved in this procedure

### 2 Postural Hypotension

In this condition the reflex mechanisms normally operating to maintain the blood pressure against the effect of gravity are in abeyance or show a greatly decreased sensitivity As a result a profound fall in blood pressure occurs in the standing position In patients with this syndrome Luft and Euler (1953) found that the urinary excretion of noradrenaline was con siderably below the normal range and that the output of adrenaline was also depressed but to a lesser degree Luft and Euler (1953) have suggested that in patients with postural hypotension the vasomotor system is unable to produce sufficient quantities of noradrenaline to maintain the homeostatic reflex activity

### 3 Muscular Work

Euler and Hellner (1952) reported that during strenuous exercise the output of both adrenaline and noradrenaline in urine rose as much as tenfold over control levels In slight or

moderate muscular work, on the other hand, the readings were generally within the normal range or were only slightly elevated On the basis of these observations it was concluded that during heavy muscular exercise both the adrenal medulla and the adrenergic nerves were in a state of increased secretory activity

## 4 Hypoglycæmia

Recent work by Euler and Luft (1952) and by Elmadjian *et al* (1956 *b*) demonstrated that the excretion of adrenaline in healthy subjects was increased approximately tenfold when hypoglycæmia was produced by the intravenous injection of insulin in a dosage of 0 1 1 u per kg body weight Insulin administration did not appreciably affect the output of noradrenaline in normal subjects In patients with acromegaly and in two cases of postural hypotension hypoglycæmia was not associated with an increase in the urinary excretion of either of the catecholamines (Euler and Luft, 1952)

## 5 Surgical Stress

Franksson *et al* (1954) found that uncomplicated surgical procedures *e g*, cholecystectomy, did not cause any marked change in the urinary excretion of adrenaline and noradrenaline On the other hand the occurrence of any complication such as pain, fear, or delayed wound healing was usually accompanied by a rise in catecholamine excretion Under such circumstances excretion values for both adrenaline and noradrenaline were abnormally high

In the cases studied by Franksson *et al* (1954) assays were also performed of the plasma concentration of 17 21 dihydroxy 20 keto corticosteroids (Porter Silber chromogens) using the method described by Nelson and Samuels in 1952 (see p 264) There was no obvious parallelism between the urinary excretion of catecholamines on the one hand and the plasma levels of corticosteroids on the other Not infrequently abnormally high plasma corticosteroid values were associated with a normal urinary excretion of adrenaline and noradrenaline

## 6 Emotional Stress

It has been claimed (Funkenstein *et al* 1952) that in certain types of emotional stress the excretion of catecholamines in

urine is abnormally high In a study on Air Force personnel Euler and Lundberg (1954) reported that the piloting of aircraft was generally associated with a rise in adrenaline excretion Noradrenaline was found in increased quantities only in pilots undertaking advanced flying Elmadjian *et al* (1956 *a*) have recently reported that the psychomotor stress involved in operating a pursuit meter caused an increased urinary output of both adrenaline and noradrenaline in healthy subjects

## 7 Adrenalectomy

Euler *et al* (1954 *a*) found that after unilateral adrenalectomy little change in catecholamine excretion occurred After bilateral adrenalectomy the excretion of adrenaline fell markedly to approximately one fifth of the normal level while the output of noradrenaline was virtually unchanged It is probable that the small quantities of adrenaline present in urine after bilateral adrenalectomy are derived from chromaffin cells present in various organs

## 8 Administration of various Hormones and Drugs

At the time of writing little reliable information is available on this subject In a patient with rheumatoid arthritis Euler and Luft (1949) showed that ACTH caused a marked fall in the output of noradrenaline but had no effect on the adrenaline excretion This finding was confirmed by Elmadjian *et al* (1956 *b*) in a study involving four healthy subjects Cortisone in a dosage of 200 mg per day produced a decrease in the quantity of noradrenaline excreted and a slight rise in the output of adrenaline (Luft and Euler 1952) The effect of insulin has been discussed above

Quantitative determinations of the excretion of adrenaline and noradrenaline in urine have been made after the intra venous administration of these compounds to healthy subjects (Euler and Luft 1951 Euler and Zetterstrom, 1955) It has generally been found that 1 to 4 per cent of the administered adrenaline and noradrenaline is recovered in the urine

Elmadjian *et al* (1956 *a*) have recently reported that the parasympathomimetic drug acetyl $\beta$ methyl choline (mecholin) causes a slight increase in the excretion of noradrenaline but does not affect the adrenaline content of urine

## THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE IN PATHOLOGICAL CONDITIONS

### 1 Essential Hypertension

In a recent study of 500 patients with this disease Euler *et al* (1954 *b*) found that the excretion of noradrenaline was within normal limits in 84 per cent of cases and was somewhat elevated in only 16 per cent These workers concluded that there was no definite evidence for an increased production of noradrenaline in the great majority of patients with essential hypertension A similar conclusion was reached by Burn (1953) and by West (1954) in a smaller series of cases Euler *et al* (1954 *b*) were unable to demonstrate any alteration in the excretion of adrenaline in patients with essential hypertension

### 2 Coronary Thrombosis

Increased excretion values for noradrenaline in acute cases of coronary thrombosis have been reported by Forssman *et al* (1952) These workers found abnormally high levels in nine out of fifteen patients studied Forssman *et al* (1952) and Nuzum and Bischoff (1953) showed that in a proportion of cases the excretion of adrenaline in urine was also raised

### 3 Phæochromocytoma

This tumour which is also called a *chromaffinoma* is associated with hyperfunction of the adrenal medulla and with a greatly increased excretion of catecholamines in urine Such tumours generally arise in the adrenal medulla itself but in approximately 10 per cent of cases they are found in the collections of chromaffin tissue situated along the abdominal aorta as far down as its bifurcation The clinical features pathology and differential diagnosis of phæochromocytoma have been recently reviewed by Goldenberg (1951) Cahill (1953) Rosenheim (1954) and others Chromaffin tumours account for less than 1 per cent of all cases of arterial hypertension in man They are however, of importance because they produce a form of hypertension which can be cured by surgery

Numerous investigators have shown that in patients with phæochromocytoma the urinary excretion of catecholamines is markedly increased (Engel and Euler 1950, Pekkarinen and Pitkanen, 1955, West and Taylor 1955, Euler, 1955) In

some cases the noradrenaline output has reached levels of 3,000 μg per twenty four hours while urinary adrenaline readings of over 750 μg per twenty four hours have been reported Euler (1955) has found that in a proportion of patients, the noradrenaline content of urine is so high that the urine need not be extracted and can be tested directly on the cat s blood pressure

The relative proportions of adrenaline and noradrenaline in urine may sometimes be of value in determining the site of the lesion (Euler, 1955) When the output of noradrenaline is very high and the excretion of adrenaline relatively normal the tumour tends to arise from the chromaffin tissue along the abdominal aorta On the other hand a tumour arising in the adrenal medulla itself tends to cause a greatly increased urinary excretion of both catecholamines

ESTIMATIONS IN BLOOD AND IN TUMOUR TISSUE —Lund and Møller (1951) and Euler *et al* (1953) demonstrated that the blood concentration of catecholamines in patients with phæochromocytoma is markedly elevated Lund and Møller reported blood concentrations of noradrenaline of 2 1 to 2 4 μg per 100 ml while Euler *et al* (1953) found levels between 3 4 and 3 8 μg per 100 ml In normal individuals the blood concentration of noradrenaline is generally less than 0 2 μg per 100 ml

The concentration of catecholamines in phæochrome tumours has been studied by—among others—Holton (1949) and West (1955) According to Holton (1949) such tumours contain relatively large quantities of noradrenaline and comparatively small amounts of adrenaline The total amounts of the two catecholamines have been estimated as 5 to 15 mg per g tumour tissue whereas normal adrenal tissue contains approximately 1 mg per g of these substances The percentage of noradrenaline in phæochrome tumours has varied from 15 to 97 per cent (West, 1955)

## SUMMARY AND CONCLUSIONS

Adrenaline and noradrenaline can be estimated in body fluids either by biological or by chemical methods It is probable that in the future improved chemical methods depending on fluorimetry will largely replace bio assay techniques

## THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE IN PATHOLOGICAL CONDITIONS

### 1 Essential Hypertension

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Extracts containing catecholamines should be purified by chromatographic methods Techniques involving either column or paper chromatography are suitable for this purpose

It is generally believed that the main source of the nor adrenaline found in urine is the adrenergic nerves Adrenaline is produced mainly by the adrenal medulla

In normal subjects the concentration of catecholamines in blood is very low and their presence cannot be detected by existing methods The urinary excretion of both adrenaline and noradrenaline is usually increased by heavy muscular work, by emotional stress and by surgical procedures in which complications supervene The production of hypoglycæmia increases the output of adrenaline but does not affect the excretion of noradrenaline

In patients with essential hypertension the urinary excretion of catecholamines in urine is generally within the normal range In cases of phæochromocytoma markedly raised excretion values are encountered and this finding is of clinical importance in the diagnosis of patients with this type of tumour

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international standard was established in 1936 by the Health Organisation of the League of Nations The international unit was defined as the amount of activity present in $\frac{1}{22}$ mg of this preparation

## METHODS OF ASSAY OF INSULIN

Until now only biological methods of assay have proved satisfactory for the estimation of insulin Various chemical procedures have been proposed from time to time but these have been found to be relatively non specific

The two bio assays which have been most widely used for comparing the relative potency of samples of insulin have been those depending on the ability of the hormone to produce convulsions in mice and to cause hypoglycæmia in rabbits Unfortunately neither of these procedures is sufficiently sensitive to detect the very small quantities of insulin present in body fluids and for this reason the techniques will not be discussed in this chapter For details regarding the selection and preparation of the animals and the design and performance of these tests the reader is referred to articles by Marks and Pak (1936) Hemmingsen (1939) Fieller (1940) Burn *et al* (1950) and Smith (1950)

Within recent years more sensitive assay methods for insulin have been developed and by use of such techniques attempts have been made to measure the concentration of the hormone in blood in health and disease Consideration will be given to the following three methods the first two of which resemble one another very closely —

1 The test depending on the fall in blood sugar in adreno-demedullated alloxan diabetic hypophysectomised rats (Anderson *et al* 1947) The originators of this test refer to animals prepared in this way as ADH rats
2 The test depending on the fall in blood sugar in alloxan diabetic hypophysectomised adrenalectomised rats (Bornstein 1950) These animals have been termed ADHA rats
3 The test depending on the increase in glucose uptake by the isolated rat diaphragm (Groen *et al* 1952 Randle 1954 *a b*)

## CHAPTER XIV

# *Insulin*

## INTRODUCTION

THIS is the hormone which is secreted by the islet tissue of the pancreas In 1889 Mering and Minkowski showed that the pancreas played an important role in carbohydrate metabolism, but it was not until 1922 that Banting and Best succeeded in preparing a pancreatic extract which was capable of relieving the symptoms of diabetes mellitus in man and animals The term insulin was given to the active principle present in pancreatic extracts

### 1 Chemical Nature of Insulin

This subject has been recently investigated by Sanger and his colleagues in Cambridge and by Craig and his associates in the United States (Sanger, 1949 *a, b,* Craig *et al*, 1950, Harfenist and Craig 1952, Ryle *et al*, 1955) Ox insulin is a protein with a molecular weight of approximately 6 000 The molecule is composed of two polypeptide chains joined together by the disulphide bridges of three cystine residues The sequence of the amino acids in the two polypeptide chains has now been determined for ox, sheep and pig insulins (Ryle *et al*, 1955, Brown *et al*, 1955)

Some years ago insulin was isolated in crystalline form According to Cohn *et al* (1941) the zinc content of insulin varies from 0 3 to 0 6 per cent, depending on the *p*H of crystallisation Insulin has a high sulphur content mainly in the form of cystine The hormone is soluble in water it is rapidly destroyed by pepsin and trypsin

### 2 The International Standard for Insulin

The standardisation of insulin has been studied in great detail Accurate standardisation of the hormone is of great importance in the treatment of diabetic patients The present

international standard was established in 1936 by the Health Organisation of the League of Nations The international unit was defined as the amount of activity present in $\frac{1}{2}$ mg of this preparation

## METHODS OF ASSAY OF INSULIN

Until now only biological methods of assay have proved satisfactory for the estimation of insulin Various chemical procedures have been proposed from time to time but these have been found to be relatively non specific

The two bio assays which have been most widely used for comparing the relative potency of samples of insulin have been those depending on the ability of the hormone to produce convulsions in mice and to cause hypoglycæmia in rabbits Unfortunately neither of these procedures is sufficiently sensitive to detect the very small quantities of insulin present in body fluids and for this reason the techniques will not be discussed in this chapter For details regarding the selection and preparation of the animals and the design and performance of these tests the reader is referred to articles by Marks and Pak (1936) Hemmingsen (1939) Fieller (1940) Burn *et al* (1950) and Smith (1950)

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3 The test depending on the increase in glucose uptake by the isolated rat diaphragm (Groen *et al* 1952 Randle 1954 *a b*)

**1 Tests in 'ADH' Rats**

In the method described by Anderson *et al* (1947) adrenal demedullation was performed just after weaning When the animals had reached a body weight of from 225 to 250 g they were rendered diabetic by the intraperitoneal administration of alloxan The diabetes was controlled by protamine zinc insulin Hypophysectomy was performed when the weight of the animals had returned to the pre diabetic level In test animals so prepared a dose of as little as 0 125 milliunits of insulin produced a significant fall in blood sugar Over a relatively wide dosage range there was a linear relationship between the logarithm of the dose of insulin and the effect on blood sugar Anderson and Long (1948) have used this technique to study the effects of growth hormone, thyroxine and adrenocortical hormones on the blood insulin concentration in rats

**2 Tests in 'ADHA' Rats**

The method of Bornstein (1950) is a modification of that of Anderson *et al* (1947) Adult rats were made diabetic by the intravenous injection of alloxan, subsequently they were subjected first to hypophysectomy and then to bilateral adrenalectomy The end point of the test again depended on the reduction in blood sugar Bornstein (1950) has claimed that the technique will detect 0 05 milliunits of insulin Bornstein and Lawrence (1951 *a*, *b*) have used this assay method to measure the blood insulin concentration in diabetics Their results will be discussed later in this chapter

It must be emphasised that tests in ADHA rats present numerous technical problems Such animals are difficult to prepare and difficult to maintain Various workers including Best (1953) and Peden (1955) have been unable to use them for the quantitative determination of insulin in blood Randle (1955) considers that assays conducted in ADH and 'ADHA rats are not completely specific for insulin and probably do not give a true estimate of the insulin content of blood plasma

**3 Rat Diaphragm Test**

This method was described by Groen *et al* (1952) and was applied to clinical problems in man by its originators and by

Randle (1954 *a*, *b*, 1955) The technique depends on the fact that the glucose uptake of the isolated diaphragm of the normal rat is increased when insulin is added to the fluid in which the diaphragm is suspended

The advantages and limitations of the rat diaphragm assay have been carefully studied by Randle (1955) This worker found that the method could detect as little as 0 125 milliunits of insulin Over a range of insulin concentration of 0 125 to 32 milliunits per millilitre there was a linear relationship between the glucose uptake of the diaphragm and the log concentration of insulin added *in vitro* to the incubation medium The method was shown to have a moderate degree of precision as indicated by a mean figure for $\lambda$ of 0 36 Randle (1955) has wisely emphasised that the test when applied to blood is not entirely specific for insulin It is probable that plasma contains substances other than insulin which affect the glucose uptake of the isolated rat diaphragm and that the final estimate obtained is a resultant of these various interacting factors Accordingly Randle (1955) and Randle and Young (1956) have suggested that the method determines plasma insulin activity rather than plasma insulin itself

## INSULIN ASSAYS IN HUMAN BLOOD IN HEALTH AND DISEASE

Insulin activity has not yet been detected in human *urine* The few assays which have so far been reported in patients have been conducted on untreated serum or plasma

### 1 Normal Subjects

Very little information is at present available in the literature regarding the blood concentration of insulin in normal individuals Groen *et al* (1952) using the rat diaphragm test, detected insulin activity in the serum of a small number of normal subjects Unfortunately, however these workers did not express their results on a quantitative basis In a more comprehensive study with the same assay method Randle (1955) found that the insulin activity of normal plasma ranged from 8 3 to 15 8 milliunits per millilitre and that the mean figure was 11 5 milliunits per millilitre

**2 Disease States**

(*a*) Diabetes Mellitus.—Bornstein and Lawrence (1951 *a*, *b*) have used the assay method depending on the fall in blood sugar in ADHA rats to investigate the plasma insulin levels in diabetics. These workers have claimed that such assays can be used to divide diabetics into two groups and that this classification may be important from the point of view of therapy of the disease.

The first group consists predominantly of young diabetics in whom the disease is relatively severe. Such individuals generally show marked hyperglycæmia, appreciable ketosis and rapid weight loss; they require insulin in order to survive. Bornstein and Lawrence (1951 *b*) conducted plasma insulin assays in fifteen patients of this type and were unable to demonstrate any activity by the method used.

The second group consists mainly of middle aged obese female subjects. In such patients ketosis is usually absent and weight loss does not occur; the diabetic state is easily controlled by dietetic means and insulin is generally not required. In thirteen patients in this group studied by Bornstein and Lawrence (1951 *b*) insulin activity was demonstrated in the plasma in all cases.

The findings of Bornstein and Lawrence are obviously of interest in relation to the ætiology and therapy of diabetes mellitus in man. Final confirmation of their claims will only be possible when more reliable assay methods for blood insulin become available.

(*b*) Hyperinsulinism.—At the time of writing there is little or no information on blood insulin levels in patients with this disease. In one case of hyperinsulinism reported by Groen *et al.* (1952) the serum insulin activity as measured by the rat diaphragm test, was abnormally high. This patient was subsequently found to have an islet cell tumour of the pancreas.

(*c*) Acromegaly and Gigantism.—Randle (1954 *a* 1955), using the rat diaphragm method found that the plasma insulin activity of patients with acromegaly and gigantism was significantly greater than that of normal subjects. In both conditions a reduction in plasma insulin levels was noted after pituitary irradiation. Three patients with acromegaly and diabetes showed levels above the normal range.

(*d*) Hypopituitarism.—In seven cases of this disease

studied by Randle (1954 *b* 1955) the insulin activity of plasma was significantly lower than that in normal subjects

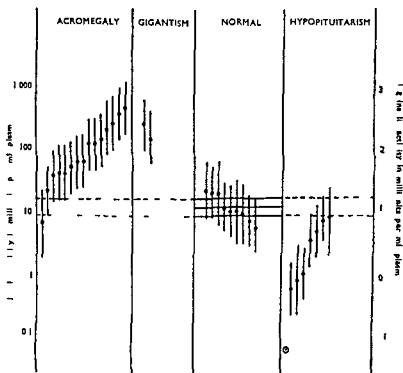



FIG 66

The insulin activity of plasma from normal subjects from acromegalic patients and from cases of hypopituitarism The assay method depended on the glucose uptake of the isolated rat d aphragm (*From Randle* 1955)

Fig 66 which is taken from a paper by Randle (1955) shows the plasma insulin activity in normal subjects and in various pathological conditions

## SUMMARY AND CONCLUSIONS

Insulin assays in blood have been conducted by the rat diaphragm test and by methods depending on the fall in blood sugar in ADH and ADHA rats None of the methods so far described is suitable for routine use in the clinical field

It has been claimed that plasma insulin assays may be of

value in the classification of patients with diabetes mellitus Proof of this interesting suggestion must await the development of more reliable assay methods

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# Index of Authors

I



J

## N



## O



## P



## Q



## R

S

T

# SUBJECT INDEX

# *Subject Index*

## C



## D

B

## H

## P